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DEVELOPING NOVEL APPROACHES TO IMPROVE RESPONSE TO T CELL BASED CANCER IMMUNOTHERAPY

Rina M. Mbofung

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**DEVELOPING NOVEL APPROACHES TO IMPROVE RESPONSE
TO T CELL BASED CANCER IMMUNOTHERAPY**

by

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TO T CELL BASED CANCER IMMUNOTHERAPY**

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DOCTOR OF PHILOSOPHY

By

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DEVELOPING NOVEL APPROACHES TO IMPROVE RESPONSE TO T CELL BASED CANCER IMMUNOTHERAPY

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Recently, T cell based immunotherapies have moved to the forefront of cancer immunotherapy with the success of Adoptive T cell therapy (ACT) and Immune checkpoint blockade. ACT, where patients are treated with tumour infiltrating T cells (TILs), conferred a clinical response rate of ~50%. Treatment with anti-CTLA4 and anti –PD1 therapy, conferred response rates of up to 50%, greatly improving the overall survival of patients with advanced melanoma amongst other cancer types. Despite the encouraging outcomes, there are relatively low response rates coupled with the delay of weeks to months before tumour shrinkage can be appreciated. Thus, understanding what tumour intrinsic pathways contribute to non-responsiveness to immunotherapies and their effect on T cells, to improve response rates, shorten time to treatment effect and developing predictive biomarkers of response are vital to the care of cancer patients.

In order to identify possible tumour intrinsic pathways that could be perturbed to improve responses to immunotherapy, a high-throughput *in vitro* screen with 850 different bio-active compounds (Selleckchem), was designed to search for agents that could either increase or decrease the resistance of melanoma tumour cells to T cell mediated killing. Paired tumour samples and TILs from melanoma patients were used to assess which compounds when used to treat the melanoma cell lines can enhance the cytotoxic activity of the TILs against the paired melanoma sample, using a flow cytometry based assay in which active caspase 3 was used as a read out of apoptosis. Heat shock protein 90

(HSP90) inhibitors amongst compounds that improved T cell mediated cytotoxicity. We show that treatment with the HSP90 inhibitor ganetespib (Synta) greatly improves T cell mediated cytotoxicity of both human and murine cancer cells lines *in vitro*. Furthermore, *in vivo* murine studies using the MC38/gp100 tumour model show that ganetespib in combination with immune checkpoint blockade, resulted in superior antitumour effect and survival compared to either treatment alone. Microarray analysis of human cell lines treated with ganetespib *in vitro* revealed an increase in interferon alpha (IFN- α) response genes including *IFIT1*, *IFIT2* and *IFIT3*. Silencing *IFIT* genes abrogated the synergy observed with ganetespib treatment and T cell mediated killing, suggesting that the IFN- α response pathway plays an important role in this combination therapy. This work highlights the importance of *IFIT* genes in response to T cell based immunotherapy and will enable the emergence of a new combination therapy of HSP90 inhibitors and anti-CTLA4 for the treatment of melanoma patients that will increase the percentage of patients responding to immunotherapy and achieving long term responses.

To understand what factors influence CD8 T cell effector function at the tumour site, we used a murine adoptive cellular therapy model in which B16, a gp100-expressing tumour cell line was implanted in mice and treated with transgenic Pmel T cells, which recognize gp100 in the context of H-2D^b. Transferred Pmel T cells were recovered from the spleen and tumour of the mice. To interrogate novel pathways which may inhibit the functions of tumour-reactive T cells at the tumour site, microarray and genome-wide gene expression analyses, were used to characterize the differential expression profiles among Pmel T cells from different groups. 720 genes were differentially expressed by T cells recovered from the tumour site, when compared with those recovered from the spleen. Amongst them, was the transcription factors Runx2, a gene whose role has not been described in T cells, to be one of the genes that were differentially expressed. In addition,

we found that when Runx2 was absent in T cells they did a better job at controlling tumour growth and vice versa. Further investigation revealed that Runx2 regulated processes in T cells such as cytokine production and differentiation status, processes which are important in controlling tumour growth. We are currently focused on understanding how Runx2 controls these processes in an effort to provide ways to improve existing immunotherapies or discover new ones.

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Chapter 1

Introduction and Background

The human body is constantly bombarded by both external and internal ‘threats’ such as pathogenic microbes, viruses and cancer. Whether the body succumbs to these threats is greatly dependent on the integrity of the host immune system. Consisting of a cooperative network of lymphoid organs, cells, humoral factors, and cytokines, the immune system can be divided into two arms: innate and adaptive immunity that work hand-in-hand. Over the years, the important role of the immune system in eradicating tumours has been well established. This concept of immunosurveillance was first proposed by Ehrlich in 1909. He posited that the immune system constantly recognized and eliminated evolving tumours before clinical manifestations could occur(1). Burnet later polished this notion with the proposal that genetic mutations occurring in somatic cells lead to the development of tumours and the immune system was vital in eradicating these mutant cells(2). This phenomenon would later be confirmed by studies from several groups, primarily through the observation that mice and humans with innate and adaptive immune-deficiencies were more likely to develop tumours (3, 4). Amongst the adaptive immune cells, T lymphocytes particularly CD8 T cells, are vital in tumour elimination as first shown in adoptive transfer experiments in murine tumour models (5-7). These studies were translated to humans with the treatment of melanoma and renal carcinoma with tumour-infiltrating lymphocytes (TILs)(8), paving the way for the development of numerous immunotherapy options for cancer patients. While there have been great strides in the development of immunotherapies, there is room for improvement as many cancer patients do not benefit from immunotherapy. Therefore, in this study considerable efforts were made, using preclinical models, to identify potential pathways or molecules in tumours and T cells that could be perturbed to improve responses to T cell based immunotherapy. It is hoped that this knowledge will inform rationale combination therapies that can provide longer lasting patient outcomes in cancer patients treated with immunotherapy. I will first introduce the general concept of the generation of an immune response and the signals that lead to optimal T cell activation. In addition, I will introduce the concept of T cell mediated antitumour immunity and how it can be suppressed. I will also summarize current strategies of T

cell mediated immunotherapy. Finally, I will focus on the known and emerging underlying mechanisms through tumours cells be unresponsive to T cell mediated immunotherapy.

Generation of a CD8 T cell mediated immune response

Although an effective CD8 T cell antitumour immune response has unique attributes, a great deal of our current knowledge on the generation of a CD8 T cell response has come from the study of acute viral infections. Following an encounter with antigen, naïve CD8 T cells follow a classical three phase response(9). Phase 1 begins with initial activation characterized by simultaneous clonal expansion of antigen specific cells and procurement of peripheral tissue homing capabilities, effector cytokine release, and cytotoxic activity. Phase 2 involves prompt, apoptosis induced contraction of antigen specific effector T cells. Finally, phase 3 involves the development of a persistent population of antigen experienced cells known as memory T cells. A comparable response has also been observed *in vivo* when tumour specific T cells are stimulated with a vaccine, accompanied by adjuvants and immunopotentiators(10). Important attributes that distinguish the memory CD8 T cells in phase 3 include increased precursor frequency compared to naïve cells, antigen independent self-renewal via homeostatic proliferation sustained by the cytokines interleukin 7 (IL7) and IL15, in addition to the rapid acquisition of effector functions and clonal proliferation following antigen re-stimulation (11-13). These characteristics are jointly known as the hallmarks of immunologic memory providing the host with long-lived protection from future pathogenic encounters. CD4 T helper cells (Th) are vital in the development and maintenance antigen specific memory CD8 T cells. As such, the ability of memory CD8 T cells to function, persist and effectively control a secondary challenge is impaired in the absence of Th (14, 15). Although tumour reactive precursor CD8 T cells are present in cancer patients, they fail to control and eliminate tumours. Studies over the years have shown these cells to be anergic or exhausted as a result of several immunosuppressive factors present within the host. An

understanding of these factors have informed the development of several T cell based immunotherapies for the treatment of cancer.

The initiation of T cell responses

Immune cells function in a precisely controlled sequential manner to provide protective immune responses. As such T cell activation is a tightly regulated process. The interaction between T cells and antigen presenting cells (APCs), including dendritic cells (DCs), B cells and macrophages, is central to the initiation of a T cell response. This interaction provides a platform for signal exchange between the two cell types. Adequate T cell activation requires the integration of antigen-dependent signals, costimulatory signals and appropriate cytokine stimulation. The mechanism of T cell activation was previously described as a two-signal model involving only antigen-dependent signals and antigen-independent costimulatory signals. With the discovery that cytokines are essential for optimal activation, differentiation, function and generation of memory this model has now been revised and is now known as a three-signal model (Figure 1).

Establishment of the two-signal model

Brescher and Cohn, are credited with the concept of the two-signal model for T cell activation as they attempted to explain self-tolerance in the periphery(16). They postulated that activation of thymic precursor cells requires the integration of antigen-mediated interactions with other non-antigen specific pathways and that interaction of antigen alone with precursor cells could lead to anergy. Their original paper lacked supporting data but over the years, their model has been confirmed and refined by many experimental observations. The first report supporting the two-signal model came from Lafferty *et al.* They compared allogenic reactions between different strains of the same species and strains from different species and found that allogeneic reactions are much higher between different strains within a species compared strains from different species(17). To explain these unexpected result, they proposed a model of cell interaction which

incorporated Brescher's second signal model, suggesting that this second signal was a species-specific costimulatory signal. While TCR/MHC interactions comprise the first signal in the two-signal model(18), the second signal was first shown to be provided by surface molecules on APCs(19, 20). Investigators observed that even though the antigenic signal was intact in T cells as evidenced by increased intracellular Ca^{2+} flux, both murine and human T cell clones were not activated by peptide-pulsed, metabolically inactive APCs or purified MHC molecules on artificial membranes. These observations suggested that the lack of activation of murine and human T cell clones under these conditions was caused by other defects of the APCs. In the early 90's, Janeway and Matzinger provided additional theories concerning the expression of inducible accessory molecules on APCs. Janeway pointed out that quiescent APCs lack the ability to provide help for T cell activation following the observation that full T cell activation required the complete Freund's adjuvant (CFA) for peptide or protein challenges. He concluded that, to induce an immune response, quiescent APCs must be activated by microbial products similar to those in CFA(21). Toll-like receptors (TLRs) were soon identified confirming and expanding upon Janeway's theory. Interaction of TLRs on APCs with pathogen-associated molecular patterns (PAMPs) derived from microbial products, leads to the surface upregulation of accessory molecules on APCs, now known as costimulatory molecules. These molecules provide the second signal of the two-signal model for T cell activation. This theory, however, only partially explained T cell activation in infectious diseases. It failed to explain other common immune responses, such as immune responses related to transplantation and tumours, and autoimmune responses. Matzinger then posited the danger signal model where, danger signals released by injured cells activated quiescent APCs, subsequently increasing the expression of costimulatory molecules(22). Many types of danger signals have since then been identified including mammalian DNA, RNA and heat-shock proteins, as well as PAMPs on bacteria(23).

Signal one: Antigen-dependent signal

The recognition of the peptide-MHC (pMHC), present on the surface of APCs, by the T cell receptor (TCR) provides the initial signal and is required for T cell activation indicating that T cells are stimulated in an antigen-specific manner. Expressed on the cell surface of T cells, TCRs consist of two transmembrane units, TCR- α and TCR- β . These units are rearranged during the process of T cell development in the thymus. A cluster of genes on chromosome 6 in humans and 17 in mice, encode for MHC molecules expressed by APCs. MHC class I and MHC class II are two major forms of these polymorphic membrane-bound glycoproteins interacting with receptors on CD8 T cells and CD4 T cells, respectively. As such, mature T cells can be classified into CD4 T helper (Th) cells and CD8 T cells (CTLs) based on the expression of these two receptors. The binding of pMHC complexes to TCRs delivers activation signals transmitted into the T cell cytoplasm via the CD3 complex (24). Following TCR engagement, tyrosine residues located in immunoreceptor-based tyrosine activation motifs (ITAMs) on the CD3 complex are phosphorylated by Lck/Fyn, belonging to the Src family of protein tyrosine kinases. ZAP70 then binds to the phosphorylated CD3 complex via its SH2 domain, and gets activated by autophosphorylation. ZAP-70 then propels TCR activation via phosphorylation several molecules including Vav, PLC γ and SLP-76 leading to the initiation of downstream signaling cascades. Finally, TCR engagement induces the activation of several pathways controlling T cell effector functions, such as the NFAT and NF- κ B pathways (25).

Signal two: Co-stimulation

The second signal required for optimal T cell activation is co-stimulation. The prototypic molecule that delivers the second signal for T cell activation is the co-stimulatory receptor CD28, a member of the immunoglobulin (Ig) superfamily of receptors. CD28 is constitutively expressed on both naïve and activated T cells(26). Following TCR ligation, CD28 on the T cells interacts with

CD80/CD86 on APCs. This interaction induces the phosphorylation of tyrosine residues on CD28, recruiting the SH-2 domain containing kinases, PI3K and Grb2. CD28 signaling amplifies the magnitude and duration of T cell responses, leading to T cell proliferation, differentiation and upregulation of survival genes such as BCL-2 and Bcl-XL(27). This signal is critical as lack thereof can lead to T cell anergy. Another member of the Ig superfamily which enhances T cell responses is, Inducible co-stimulator (ICOS). Mice with defects in ICOS and its ligand, ICOSL, signaling have severely impaired primary T cell responses, especially Th2 responses(28). Additional costimulatory molecules include CD27/CD70, OX40/OX40L, 4-1BB/4-1BBL, HVEM/LIGHT and CD40/CD40L, belonging to the tumour necrosis factor (TNF) / TNF receptor (TNFR) superfamily(29). In general, members of the TNF/TNFR family stimulate T cell proliferation and cytokine production following initial T cell activation.

Following T cell activation is the upregulation of inhibitory receptors such as CTLA-4 which dampen T cell activation to prevent autoimmunity once the 'threat' is cleared. Additional co-inhibitory receptors include PD1, LAG3, TIM3 and BTLA(30). Ultimately, the fate of T cell activation is determined by the integration of multiple co-receptors in T cell-APC focal synapses. In the context of co-stimulatory receptors such as OX40, 4-1BB and ICOS, the outcome is a favourable sustained T cell response characterized by increased proliferation, differentiation, cytokine production, survival and memory. Whereas co-inhibitory receptors such as CTLA-4, PD1 and LAG-3 could result in the opposite effect, in addition to T cell anergy and exhaustion. In summary, all of these discoveries shed light on the fact that T cell activation is more complex than previously thought. T cell activation is the result of the binding of the TCR with pMHC complex, and interactions of a variety of costimulatory molecules expressed on T cells and APCs. In addition to the nature of TCR stimulation, the efficiency of T cell activation is controlled by a large network comprised of a variety of costimulatory molecules (Figure 2).

Signal three: Cytokine stimulation

While the two-signal model was the central concept and foundation for T cell activation, it is now known that appropriate cytokine stimulation is also required for optimal T cell activation.

Mescher and Curtsinger first demonstrated this concept using *in vitro* studies(31, 32). Beads conjugated with pMHC complexes and costimulatory molecules (artificial APCs) were used to stimulate purified CD8 T cells and results indicated that signal 1 and 2 were sufficient to induce T cell proliferation and IL2 production but not cytotoxic activity. Addition of the cytokine IL12 provided the third signal for cytotoxic activity of the CD8 T cells. Similarly, their *in vivo* studies also confirmed this finding as the presence of only signal 1 and 2 and absence of IL12 resulted in peripheral tolerance of CD8 T cells(33). This phenomenon was also observed in other models. Studies from Filatenkov and colleagues evaluating the coordinated efforts between CD4 and CD8 T cells in the development of an effective cytotoxic T lymphocyte (CTL) response, showed that Th cells conditioned dendritic cells to produce IL-12 and that IL-12 was necessary to support development of CD8 T cell effector functions and graft rejection(34). Type I interferons (IFN- α and IFN- β) and IL21 were subsequently identified as additional cytokines that can also provide signal 3. Unlike IL12 and IFN- α/β , IL21 stimulation results in the development of cytotoxic activity but not IFN- γ upon re-stimulation. The gene expression pattern of cells stimulated with IL12 and IFN- α/β along with signal 1 and 2 was evaluated by Agarwal and colleagues(35). When naïve cells were stimulated for 3 days with artificial APCs, transient gene expression changes were observed which reverted to naïve levels by 72 hours. However, in the presence of either IL12 or IFN- α/β , the changes in gene expression increased and persisted at 72 hours, as well as additional changes consistent with the induction of a critical sustained differentiation program in CD8 T cell function and memory. Many of the genes regulated by IL12 and IFN- α/β such as granzymes, IFN- γ , CD25, OX40, and Bcl-3, are involved in effector functions, proliferation and co-stimulation, survival, trafficking, and migration of T cells.

Therefore signal 3 provided by the cytokines IL12 and IFN- α/β play a critical role in transplantation, tumour biology and vaccine development as well as other T cell mediated immune responses.

T cell mediated antitumour response

As mentioned above, adoptive transfer experiments in murine models informed the process of elimination of cancer cells by T cells. Particularly the CD8 T cell subtype capable of cytotoxic activity. The positive clinical outcomes of cancer patients, which received *in vitro* expanded tumour-reactive T cells, provided powerful proof that CD8 T cells can mediate objective cancer regression (36). As such, much more effort has been dedicated to their role in tumour immunosurveillance compared to CD4 T cells. Moreover, the major histocompatibility molecule (MHC) class I, indispensable for CD8 T cell activation, is expressed by most cells, including some cancer cells. Activated CD8 T cells can therefore, recognize and lyse tumour cells. Activated CD8 T cells or cytotoxic T lymphocytes (CTLs) kill tumour cells via two major mechanisms: secretion of cytotoxic granules and the Fas/FasL death pathway (37). Granzymes and perforin, which are pre-synthesize cytotoxic proteins stored in lysosomes, are released upon CD8 T cell activation to lyse the tumour cells. The release of these cytotoxic proteins is targeted to tumour cells in a specific manner such that they are not released into the extracellular milieu. They cleave critical substrates, which initiate apoptosis or DNA fragmentation in tumour cells. The importance of granzymes and perforin in antitumour immunity was demonstrated using deficient mice. Compared to wild-type mice, perforin deficient mice are more susceptible to methylcholanthrene induced tumours, while granzyme A and granzyme B deficient CTLs were unable to induce DNA fragmentation(38). Furthermore, blocking the granzyme B/perforin pathway via overexpression of the serine protease inhibitor PI-6, is associated with resistance to CTL-mediated tumour clearance (39, 40). In an independent study, the ligation of Fas ligand (FasL), expressed on the surface of CTLs, with the Fas receptor on tumour cells triggered apoptosis through the classical

caspase pathway (37). In addition to contact dependent cytotoxic mechanisms through cytotoxic granules and Fas/FasL interactions, CTLs can also kill tumour cells through contact independent mechanisms. This is achieved through release of several cytokines including interferon gamma ($\text{IFN-}\gamma$) and tumour necrosis factor alpha ($\text{TNF-}\alpha$). $\text{TNF-}\alpha$ binds to receptors expressed by tumour cells inducing activation of the caspase pathway, leading to tumour cell apoptosis. $\text{IFN-}\gamma$ interacts with specific receptors, ubiquitously expressed on all nucleated cells. This interaction initiates the JAK-STAT pathway, activating the expression of several genes including MHC molecules, the antigen processing and presentation machinery, as well as increasing antigen presentation(41). In addition, $\text{IFN-}\gamma$, through Fas/FasL upregulation, can induce apoptosis in tumour cell via caspase-1. $\text{IFN-}\gamma$, together with $\text{TNF-}\alpha$ trigger the production of reactive oxygen species (ROS) and NO, which might also inhibit the tumour growth (42). Chemokines produced by CTLs, such as RANTES, also contribute to the recruitment and activation of innate immune effector cells to control tumour growth (43).

Suppression of the T cell mediated antitumour immune response

The cancer immunity cycle is series of sequential steps involved in the generation of an effective anti-tumour immune response (44, 45). Beginning at the tumour site, APCs capture and process antigens released by oncogenesis. These antigens vary in type ranging from differentiation antigens, overexpressed antigens to mutational neoantigens(46-48). Following migration to the lymph nodes, APCs present the processed antigens to T cells in the context of MHC, thereby priming and activating effector T cell responses. T cell priming has conventionally been thought to occur exclusively in tumour-draining lymph nodes. However, spontaneously organized tertiary lymphoid organ structures can be also found within tumours signifying that T cell education may occur within the tumour bed(49). Activated T cells migrate to the tumour bed where the TCR recognizes its cognate peptide in the context of MHC. Recognition of tumour cells leads to their T cell mediated lysis. This further releases additional TAAs thereby propagating the cycle.

Interruption of the cycle at any step can impede the generation an antitumour immune response. Over the years, many tumour related factors, that impede the generation antitumour responses, have been identified(45). Given that TILs are such significant prognostic markers for tumour progression across multiple tumour types, understanding the processes involved in their suppression is essential to the improvement of current therapeutic strategies and development of novel therapeutic strategies. In this section, I will outline direct and indirect ways in which tumours suppress several steps in the generation of an effective antitumour immune response; these range from generation of tumour-reactive T cells to their homing, recognition and killing of tumour cells.

Suppression of T cell priming

As reviewed above the first two steps of the cancer immunity cycle involve APCs which process and present tumour antigens to T cells, as well as provide essential co-stimulatory signals. As such, APCs are particularly important for the coordination of an anti-tumour immune response. As professional APCs, DCs are the major type cell type that present TAAs to both B cells and T cells, generating an antigen-specific antitumour response. Optimal priming of tumour-reactive T cells depends on the apt differentiation and maturation of DCs, which provide effective antigen stimulation and co-stimulation. The tumour microenvironment can however be suppressive to the differentiation and maturation of DCs(50, 51). In mice and humans, defective dendritic cell function is often associated with deregulation of DC maturation. Mature DCs are located in peripheral lymphoid organs such as the spleen and lymph nodes. They express robust levels of MHC and costimulatory molecules and activate tumour-reactive T cells to initiate a T cell mediated antitumour immune responses. In contrast, immature/partially differentiated DCs are located in the periphery, express intermediate amounts of MHC molecules, low levels of costimulatory molecules as well as high levels of immunosuppressive cytokines and inhibitory molecules. They inefficiently process and present TAAs to T cells inducing the loss of function of

tumour-reactive T cells (52). All-in-all immature DCs mediate immune tolerance to tumours, inducing anergy of effector T cells and/or favouring the expansion of T regulatory cells (Tregs) in the lymph nodes or at tumour sites (53, 54). Till date several tumour secreted factors which suppress DC maturation have been identified. Vascular endothelial growth factor (VEGF) produced by tumour cells was one of the first factors reported to induce immature/partially differentiated DCs (51). In addition to suppression of DC maturation, VEGF can also induce high levels of PDL1 expression thereby also impairing DC function(55). Additional tumour derived factors that disrupt DC maturation and function include macrophage colony stimulating factor (M-CSF), Interleukin 6 (IL6), IL10, transforming growth factor beta (TGF- β) which inhibit differentiation of DC (56-58), as well as physiological stimuli such as hypoxia and lactic acid(59-61). Finally, immature DCs fail to secrete appropriate chemokines important in recruiting effector cells to the tumour(62). In summary, normal DC differentiation, maturation and function are essential components of T cell priming and disruption of this process via tumour-mediated immune suppression leads to tumour immune tolerance. Current strategies aimed at relieving this immune suppression include blocking these aforementioned tumour secreted immunosuppressive molecules, stimulating DC maturation via administration of TLR agonists or CD40 agonistic antibodies, as well as generating potent DC-vaccines *ex vivo*.

Suppression of T cell homing

Chemokines play a critical role in T cell trafficking or homing to the tumour. As such, tumours disrupt normal chemokine expression, greatly contributing to impaired T cell trafficking. Tumours with a great number of TILs express high levels of established T-cell-attracting chemokines, including chemokine (C-C motif) ligand 2 (CCL2), CCL3, CCL4, CCL5, chemokine (C-X-C motif) ligand 9 (CXCL9), and CXCL10(63). In the tumour milieu, these chemokines can also be induced by IFN- γ secreted by T cells. From observations in murine models, it is thought that a few T cells initially infiltrate tumours along a chemokine gradient, followed by a large influx of both specific

and nonspecific T cells as a result of a positive feedback loop that amplifies these T cell attracting chemokines(64, 65). In tumour cells, it is well known that aberrant post-translational modifications occurs in expressed chemokines. Changes in their cleavage, deamination and glycosylation results in dramatically altered activities of expressed chemokines (66, 67). For instance, reactive oxygen species (ROS) can induce nitrosylation of CCL2, an important chemokine for the recruitment of CTLs to the tumour site(68). This post translational modification abrogates the ability of CCL2 to attract tumour-specific CTLs, instead attracting myeloid-derived suppressor cells (MDSCs) to the tumour bed(69). Additionally proteolytic processing of CXCL11, an important chemokine that recruits CXCR3 expressing effector T cells, could be significantly altered. This weakens CXCL11 binding and signaling, greatly decreasing lymphocyte homing (63, 64, 67). Furthermore, CCL22 expression in ovarian and breast cancer can result in recruitment of Tregs within tumours forming an immunosuppressive microenvironment (70). Hence, the deregulation of chemokine expression is an important tumour immune escape mechanism.

Suppression of T cell extravasation

In addition to the chemokine gradient, the vascular endothelium also plays an important role in T cell trafficking, particularly transmigration of T cells through the vascular endothelium into target tissue in a process known as extravasation. T cell extravasation through the endothelium into the tumour is a multi-step process that includes rolling and adhesion to endothelial cells and subsequent diapedesis. The tumour endothelium can, however, be prohibitive in nature to T cell transmigration, depending on the type and quantity of adhesion molecules expressed (36, 71, 72). Key adhesion molecules important for extravasation include intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), expressed on the endothelium. ICAM-1 and VCAM-1 are known to be upregulated by soluble factors such tumour necrosis factor- α (TNF- α) often expressed within the tumour microenvironment, albeit in low amounts by tumour cells themselves(73). TNF- α is a known activator of endothelial cells and T cell adhesion.

However, in the presence of angiogenic promoting factors such as basic fibroblast growth factor (bFGF) or VEGF, TNF- α stimulation is unable to induce expression of ICAM-1 and VCAM-1 on endothelial cells(74, 75). In addition to VEGF, the presence of endothelins and their receptors within the tumour microenvironment is detrimental to the expression of adhesion molecules. Frequently upregulated in a number of cancers including ovarian, colon, breast, prostate and renal cancer endothelin (ET) peptide ligands ET-1, -2, -3, and -4 are potent regulators of endothelial cell biology(76-78). They are involved in autocrine and paracrine loops that promote angiogenesis, proliferation, protection from apoptosis, vasculogenesis, invasion and metastatic dissemination of tumours. Through interactions with VEGF, endothelins regulate several aspects of angiogenesis, including endothelial cell proliferation, migration, invasion, vessel formation, and neovascularization. The suppressive effects of VEGF and endothelin can be commonly induced by the highly reactive free radical nitric oxide (NO)(74, 79). NO decreases T cell-endothelial interactions via downregulating the expression of the important adhesion molecules Pselectin, ICAM-1, and VCAM-1. As such, inhibition of the NO-producing enzyme, nitric oxide synthase (NOS), enhances both the rolling and adhesion of CD8 T cells on tumour vasculature. Independent of adhesive mechanisms, the tumour vasculature can also influence the nature of T cell infiltration in tumours. Endothelial cells can, for example, express a number of molecules such as FasL and TNF-related apoptosis-inducing ligand (TRAIL) which can interact with corresponding receptors on effector CD8 T cells leading to their suppression and death(80, 81). Furthermore, immunosuppressive molecules such as TIM-3, IL-10, TGF β , PDL1, PDL2, B7-H3 and PGE2 can also be expressed by the tumour endothelium(76, 82-88). Therefore, the role of tumour endothelial cells is mostly immunosuppressive and is maintained by tumour cells through paracrine mechanisms.

Suppression of T cell interaction and recognition of tumour cells

Once CD8 T cells emerge into the tumour bed, they can encounter additional immunosuppressive cell populations within the tumour microenvironment. T regulatory cells (Tregs) are one of such cell types encountered(89-91). They can be actively recruited and locally expanded to effectively suppress T cell effector functions. As such, infiltration of Tregs is frequently associated with a poor prognosis, although their presence may also be indicative of an ongoing immune response. Characterized by the markers, CD4⁺ CD25⁺ FoxP3⁺, Treg cells can be divided into natural Tregs (nTregs) and inducible Tregs (iTregs)(92). nTregs are thymically derived and maintained peripherally by TGF- β , while iTreg are induced from naive CD4⁺ T cell precursors periphery. A number of studies indicate that Treg cells accumulate within tumours in a clonally restricted manner and are specific for tumour antigens. Treg cells are therefore, activated in an antigen-specific manner and can suppress T cell function through both specific and nonspecific mechanisms(91, 93). Tregs cells can be actively recruited by chemokines such as CCL22 secreted by tumour cells and tumour associated macrophages(70). In addition to recruiting nTregs, the tumour microenvironment favours the continued expansion of nTregs as well as the generation of iTreg cells via IL-10, TGF- β and adenosine derived from either tumour-resident immunosuppressive DCs and Tie-2⁺ monocytes (TEMs) or tumour cells(94). Cytokines such as TGF- β , IL-10, and IL-35, secreted by Tregs suppress effector T cell expansion and effector cytokine secretion. Another mechanism through which Tregs suppress effector cells is through competitive consumption of IL2 in the tumour microenvironment, thus limiting effector T cell expansion and function(95). Tregs express high levels of the IL-2 receptor alpha (IL-2R α , also known as CD25) and hence have higher affinity for IL2. While IL2 signaling on Treg cells is not required for their suppressive function, IL2 is required to maintain their metabolic homeostasis and competitive fitness *in vivo*. Thus, Treg cells depend on paracrine support from T effector cells, which secrete IL-2, for expanding and maintaining local tolerance. In addition, Tregs can directly

kill T cells via TRAIL or granzyme B mediated cytotoxicity (96, 97). Finally, Tregs can crosstalk with DCs, inducing the expression of TGF- β , IDO and IL-10 expression from DCs through direct interactions(91).

While a number of suppressive myeloid lineage cells have been identified within tumours, myeloid derived suppressor cells (MDSCs) are the best described. Often found in great numbers within tumour and in circulation of cancer patients, they are potent inhibitors of effector T cell functions(98). Recruited from the bone marrow, MDSCs are expanded in the peripheral blood through interactions with BV8 and endocrine-gland-derived VEGF (EGVEGF)(99). Once in circulation, MDSCs can be recruited a number of chemokines, including CCL2, CXCL5, CXCL12, and stem cell factor (SCF) to the tumour site(100). Within the tumour, MDSCs suppress T cells through the production of IL10, TGF- β , arginase I, as well as reactive oxygen species (ROS). Furthermore, MDSCs can also expand Treg cells within the tumour microenvironment (59, 86). Nevertheless, studies have shown that MDSCs can be phenotypically plastic. They can acquire the features of tumour-rejecting monocytes and even APCs if the right conditions are met. For instance, the cytokines IFN- γ and IL12 have been shown to convert MDSCs into APC-like cells that activate and enhance the functions of T cells *in vitro* and *in vivo* (101, 102). Therapies that deplete MDSCs or change their phenotype to a more favourable one are attractive for cancer treatment.

Suppression of T cell mediated killing of tumour cells

Finally, once T cells successfully make their way through the barriers of the tumour vasculature and stroma, they can face additional immunosuppressive factors that interfere with effective recognition and/or killing of tumour cells. As previously described, T cells rely greatly on pMHC-TCR interactions in order to recognize targets and perform their effector functions. It is well established that that tumours express protein products or peptides that can be recognized by the immune system as "non-self". These peptides are derived from a range of proteins including

overexpressed self-proteins (including cancer testis and other immune-privileged site antigens) to novel mutational epitopes resulting from nonsynonymous somatic mutations (47, 103). T cells recognizing these tumour associated peptides have been identified in melanoma as well as other cancer types. Tumours have however developed mechanisms to avoid T cell recognition by T cells through loss of immunogenic peptides from the tumour surface. This could be accompanied by loss of expression or downregulation of the antigen processing and presentation machinery, as well as downregulation or complete loss of MHC I expression(104). Selective pressure from mutation, genetic loss, or epigenetic silencing could be responsible for this observation. In addition, tumour intrinsic oncogenic signaling could also contribute to this effect as inhibition of certain pathways can increase MHC I and surface antigens on tumour cells (104, 105). This aberration in antigen processing and presentation is associated with poor prognosis of disease and poor clinical outcomes.

Tumours can also express surface molecules including the TNF family members FasL and TRAIL that can directly kill T cells following interaction with the receptors on T cells(106). They can also express the ligands to co-inhibitory receptors on T cells, such as PDL1, PDL2 and B7-H4 which can suppress T cell functions arresting tumour rejection (107, 108). Furthermore, the microenvironment in close proximity to tumour cells can be quite toxic for optimal CTL function. Soluble mediators, such as IL-10, PGE2, TGF- β , histamine, hydrogen peroxide, and adenosine, secreted by tumour cells, can directly inhibit CTLs(106). Moreover, metabolic substrate deprivation as a result of competitive consumption by tumour cells and/or active depletion by enzymes such as IDO and arginase can further diminish T cell effector function(109). Finally, the hypoxic conditions and the relatively lower extracellular pH reminiscent of the tumour interstitium can negatively affect CTL function(90).

T cell mediated cancer immunotherapy

Given the crucial role of tumour-reactive T cells in antitumour immune responses and an increased understanding of tumour suppressive mechanisms, T cell mediated cancer immunotherapy has been exploited for several decades to harness or boost the immune system to eliminate tumours. Till date many immunotherapies have been developed and successfully implemented for the treatment of various cancer types including melanoma, renal cell carcinoma, non-small cell lung cancer and lymphoma. Cancer immunotherapy can be divided into four major categories: cancer vaccines, cytokine based therapy, adoptive cellular therapy and immune checkpoint therapy.

Cancer vaccines

The concept of cancer vaccines was borrowed from the successful development of vaccines against infectious diseases. Cancer vaccines illicit recognition and destruction of tumour cells by activating the immune system. Common forms of cancer vaccines being investigated for cancer treatment include protein vaccines, whole cell vaccines, DNA vaccines, peptide vaccines, recombinant virus vaccines and dendritic cell vaccines. The identification of several differentiation antigens such as gp100 and MART-1 paved the way for the development of cancer vaccines. Additional tumour antigens used in cancer vaccines include overexpressed antigens (Mesothelin), viral antigens (HPV E6, E7), cancer-testis antigens (MAGE family) and neo-antigens (Ras)(110, 111). Although the success of cancer vaccines has been limited, the results from pre-clinical and clinical studies thus far have provided insight into how their therapeutic efficacy can be improved. For example, results from a standalone phase II study in which patients with metastatic melanoma were treated with the gp100 peptide followed by Interleukin 2 (IL2) resulted in a 42% objective clinical response providing evidence that vaccines could provide therapeutic benefit for cancer treatment. A follow up randomized phase III study where patients received either gp100 alone or gp100 followed by IL2 indicated that addition of IL2 provided additional benefit over gp100 alone

as there was a significant improvement in the overall clinical response (36). These studies not only showed the potential of cancer vaccines but the ability to be potentiated by adding additional agents. As such, studies geared towards improving cancer vaccines via finding the right adjuvants and immunopotentiators are underway(112).

Cytokine therapy

Cytokines are small proteins secreted by cells with a specific effect on the communication and interaction between cell types. The two most common cytokines used for cancer treatment are Interferon alpha (IFN- α) and IL2. IFN- α , a type I interferon, is produced by T cells, DCs, natural killer cells and macrophages. In addition to directly inducing apoptosis in tumour cells, IFN- α also stimulates anti-tumour innate and adaptive immune responses. Although the results regarding the overall survival outcomes of IFN- α therapy in clinical studies are controversial, studies indicate that IFN- α can provide disease free survival benefit when used in an adjuvant setting. However IFN- α therapy can produce substantial toxicities in patients undergoing treatment (113-116). The FDA approved cytokine IL2, was the first interleukin to be identified and cloned. This glycoprotein is mainly produced and secreted by T helper cells, although it can also be produced by activated CD8 T cells. IL2 has pleiotropic effects on several subsets of immune cells. It is vital in the differentiation, proliferation and survival of T cells, as well as promoting the production of immunoglobulins by B cells, boosting activation and proliferation of natural killer (NK) cells(117). Because of its role in the promotion of the activation, expansion and survival of CTLs and NK cells, IL2 has been used as a cancer therapy for quite some time. High dose IL-2 induces a 15% response rate leading to a 5% cure of patients with metastatic melanoma(118). Therefore, was one of the first-line treatment options for metastatic melanoma. It has also improved the therapeutic outcome when used in combination therapies with other immunotherapies such as adoptive T cell therapy and cancer vaccines(119). Other cytokines with promising therapeutic

benefit in cancer treatments include GM-CSF, IL21 and IL15. Clinical trials of evaluating their therapeutic efficacy underway.

Adoptive cellular therapy

Preclinical murine syngeneic tumour models revealed the presence tumour infiltrating lymphocytes (TIL). This discovery lead to the development of adoptive cellular therapy (ACT) which involves isolation or engineering of T cells, *ex vivo* expansion and transfer of these T cells back into patients(120). In one form of ACT TILs are isolated from resected tumour masses of patients, expanded *ex vivo* and re-infused back into patients. In the early years, the clinical responses to ACT were not encouraging. An overall objective response rate of 31% with a complete response rate of only 5.8% was observed in a clinical study where 86 patients with metastatic melanoma received autologous TILs followed by high dose IL2. To improve ACT, follow up studies employed lymphodepletion which eliminated endogenous lymphocytes, decreased competition for homeostatic cytokines and growth factors, and elimination of immunosuppressive cells (36). The objective response rate observed when patients with metastatic melanoma receiving either 2gy or 12gy total body irradiation was 52% and 72% respectively with a complete response rate of about 22% indicating the potential of ACT as an effective therapy for late stage melanoma. Although the results obtained with ACT are exciting, there are still many challenges that need to be overcome. For one, it is still unclear what the antigen specificity of TIL is and what percentage of TILs recognize the tumour. Thus, there may be expansion of non-tumour reactive or bystander TILs decreasing the effectiveness of therapy. In addition, the tumour environment can be quite immunosuppressive which could affect migration and the persistence of TILs *in vivo*. Moreover, recent studies indicate the quality of TILs infused could be improved as far as their differentiation status which could in turn affect the effector function and persistence of TILs *in vivo*. Finally, the process itself is expensive, technically

challenging and labor intensive. Notwithstanding, preclinical and clinical studies are currently ongoing to evaluate multiple strategies to improve ACT.

One strategy to overcome the antigen specificity limitation is to genetically engineer autologous TILs or peripheral blood mononuclear cells (PBMCs), via retroviral or lentiviral transductions, to express tumour associated antigen (TAA) specific TCRs. As described earlier, TCRs recognize antigens in the context of MHC or the human homolog, human leukocyte antigen (HLA). Because TAAs are 'self' antigens, their naturally occurring TCR affinities are quite weak as a result of thymic selection. Therefore, these TCRs are usually modified *in vitro* to confer higher binding affinity(121). The most advanced examples of engineered high affinity TCRs against TAAs, are the HLA-A*0201-restricted MART1 and HLA-A*01-restricted MAGE-A3 TCRs (120, 122, 123). Despite an encouraging clinical response of about 30% observed melanoma patients treated with the MART1 TCR, majority of the patients experienced severe off target toxicities in melanocytic expressing sites such as the ears, eyes and skin. Most severely, the first two patients that received the MAGE-A3 TCR expressing T cells died from cardiogenic shock as a result of the TCR recognizing an unrelated MAGE-A3 protein expressed by normal cardiac cells. These results raise a concern of unwanted cross reactivity due expression of target antigen on normal tissue. Nonetheless, cancers which express certain antigens, such as cancer testis antigens (CTA) may benefit the most from this therapy since these antigens are highly in multiple tumour types but not in normal adult tissue. While TCR expression favours generation of antigen specific T cells not restricted to TILs, there are still some hurdles to overcome. For one, HLA expression is heterogeneous amongst patients with the same cancer type, hence the engineered TCR may only be applicable to a limited number of patients with the same HLA type. Moreover, as a mechanism of immune escape, tumours are known to downregulated HLA expression rendering themselves invisible to T cells.

To circumvent HLA-restricted antitumour reactivity of T cells, T cells can be engineered to express chimeric antigen receptors (CARs). CARs combine the antigen specificity of antibodies with the cytotoxicity and survival of T cells such that, CAR-T cells recognize and kill tumour cells independently of HLA. First created in the 1980s, the first generation of CARs were constructed by linking the single-chain variable fragment (scFv) of a monoclonal antibody to the intracellular TCR signaling domain CD3- ζ (124). Results from initial clinical trials with CAR-T cells were not very encouraging characterized by mediocre expansion and survival of transferred CAR-T cells *in vivo*(125). Subsequent studies lead to the development of second and third generation CARs which included one or two co-stimulatory domains in addition to the aforementioned components, greatly enhancing CAR- T cell persistence *in vivo*(120). Till date, the most successful CAR is the CD19 targeting CAR in patients with B-cell malignancies. Similar to TCR engineered T cells, the expression level of targeted antigens on normal cells is crucial to limiting toxicity of CAR-T cells. In addition, both treatment modalities usually target a single antigen favouring tumour escape. Therefore, evaluations of bispecific CARs or combination of CARs with varying antigen specificity to prevent antigen escape are ongoing

As mentioned above one important limitation of ACT is that efficient migration of transferred TILs into the tumour bed is quite lacking. While clinical studies, using indium-111 labelled TILs, indicate that the number of adoptively transferred TILs migrating into the tumour positively correlates with clinical response(126), the trafficking efficiency of TILs can be tremendously mediocre(127). One important aspect of T cell trafficking is the ability to localize to infection sites or tissues along a chemokine gradient. In the case of an anti-tumour response, this process is enabled by expression of the appropriate chemokine receptors on T cells to facilitate localization to the tumour milieu expressing the corresponding chemokines. Evaluation of human melanoma tumours revealed a preferential expression of high levels of the chemokines CXCL1 and CXCL8, hence a bias towards high levels of monocyte/macrophage lineage infiltrates which

highly express CXCR2, the receptor to these chemokines (63, 128, 129). Borrowing a leaf from nature's book, investigators overexpressed CXCR2 in adoptively transferred T cells in preclinical murine studies and showed that this greatly enhanced the migration of adoptively transferred T cells into the tumour, leading to enhanced tumour regression(130). Clinical trials to evaluate this improvement are currently ongoing.

Finally improving the persistence and effector function of adoptively transferred TILs is crucial to improving ACT. I will expand on this topic in chapter 3.

Immune checkpoint therapy

In order to sustain an antitumour immune response, the co-receptor content in the context of T cell activation is very crucial. The tumour microenvironment could be quite a suppressive milieu, with the tumour cells themselves and myeloid derived suppressive cells (MDSCs) expressing the ligands to co-inhibitory receptors, thereby dampening T cell activation. An understanding of the importance of co-stimulatory and inhibitory pathways has led to the clinical development of modulatory antibodies targeting these receptors in order to sustain anti- tumour immune responses. In the case of co-stimulation, agonistic antibodies to the co-stimulatory TNF-receptor superfamily members 4-1BB and OX40 are the farthest along in clinical trials. While, blocking antibodies to co-inhibitory receptors CTLA4 and PD1, have been FDA approved for the treatment of multiple cancer types. I will expand upon the latter below.

Anti-CTLA4

The co-inhibitory receptor, Cytotoxic T-lymphocyte antigen (CTLA)-4, a member of the immunoglobulin (Ig) superfamily. Located on human chromosome 2q33 and mouse chromosome 1, CTLA4 is exclusively expressed on T lymphocytes (131). Unlike CD28, CTLA4 expression on T cells is activation induced (132). As such activated but not naïve T cells express CTLA4. Following TCR activation on conventional T cells, CTLA4 is sequestered from intracellular trans-

golgi network compartments to the immune synapse where it binds competitively to its ligands CD80 and CD86 on APCs with stronger affinity over its co-stimulatory Ig family member CD28 (133). Unlike CD28, CTLA4 binding inhibits IL2 secretion, induces cell cycle arrest and downregulates T cell activation. While the mechanism of action of CTLA-4 is still under debate, it is thought CTLA4 outcompetes CD28 for the ligands CD80/CD86 thereby preventing positive signaling. In addition, CTLA4 recruits the phosphatases, SHP2 and PP2A, to the immune synapse which subsequently dephosphorylate key signaling components such as ZAP70 downstream TCR activation (134, 135). Moreover, CTLA4 can confer T cell inhibition through the sequestration of CD80 and CD86 from CD28 engagement, as well as active removal of CD80 and CD86 from the APC surface.

In contrast to conventional T cells, CTLA4 is constitutively expressed on T regulatory cells (Tregs). CTLA-4 is a target gene of the forkhead transcription factor 3 (FOXP3) and can contribute to the suppressive ability of Tregs. There have however been conflicting *in vitro* and *in vivo* reports in the field concerning the contribution of CTLA4 to Treg suppression. Sakaguchi and Powrie provided initial evidence that the CTLA4 pathway could be used to elicit Treg suppression (136, 137). However, reports from early work performed by the Shevach group and others showed failure to reverse Treg suppression following CTLA4 blockade *in vitro* (138). Subsequent studies demonstrated that Tregs from CTLA4 deficient (CTLA4^{-/-}) mice retained their suppressive function *in vitro* (139). In some studies CTLA4^{-/-} Treg suppressed slightly less efficiently than wildtype Tregs, echoing the early observation that CTLA4^{-/-} Tregs showed about 50% suppression compared to about 95% suppression elicited by their wildtype counterparts (137). Moreover, studies from Tang and colleagues indicated that even though CTLA4^{-/-} Tregs were capable of suppression, the function of wildtype Treg was abrogated by CTLA4 blockade (140). This suggested that wildtype Tregs use CTLA4 to suppress but that compensatory mechanisms develop in CTLA4 deficient animals. Suppressive compensatory pathways were elegantly

demonstrated by the observation that a dual deficiency in IL-10 and IL-35 (suppressive cytokines secreted by Tregs) results in a remarkable compensatory increase in TRAIL expression and increased dependence on the TRAIL pathway for suppression *in vitro*. In *in vivo* settings, there is now overwhelming evidence supporting a role for CTLA4 in Treg suppression(140). Particularly key evidence that CTLA4 can physically remove its ligands CD80/CD86 from antigen presenting cells by a process known as trans-endocytosis, providing a mechanism for Tregs to regulate CD28 stimulation of other T cells.. All-in-all, the general consensus is that CTLA-4 plays a key role in Treg function although other mechanisms can sometimes substitute in its absence.

Known as the godfather of immune checkpoints, CTLA4 was the first immune checkpoint receptor to be targeted for the treatment of cancer, paving the way for targeting other immune checkpoint receptors(131). Because of the lack of tumour specificity for the expression ligands of CTLA4 on solid tumours, coupled with the fact that CTLA4 knockout (KO) mice displayed a hyper- and autoimmune phenotype that was lethal, the general strategy of CTLA4 blockade was unattractive. Nevertheless, reports from Allison and colleagues using preclinical models demonstrated that partial blockade of CTLA4 using antibodies could provide a therapeutic effect without overt immune toxicities(141). Immunogenic tumours treated with CTLA4 blocking antibodies as single agents, responded to treatment while poorly immunogenic tumours did not respond unless combined with a granulocyte–macrophage colony stimulating factor (GM-CSF)-transduced cellular vaccine. In addition, CTLA4 blockade lead to the depletion of Tregs from the tumour microenvironment, coupled with an increase in the expression of the co-stimulatory molecule ICOS on TILs. These findings suggested that, CTLA4 blockade could enhance endogenous antitumour immune responses, which ultimately can induce tumour regression. Based on these preclinical findings, two fully humanized antagonistic CTLA4 antibodies, ipilimumab and tremelimumab, were produced and clinically evaluated beginning in the year 2000. As single agents both antibodies produced objective clinical responses in ~10% of patients

with metastatic melanoma (142-144). However, immune-related toxicities involving various tissue sites, particularly colitis, were observed in about 25–30% of patients. In a randomized Phase III clinical trial, patients with advance melanoma were treated 15mg/kg of tremelimumab, given every three months as a single agent and compared with dacarbazine a standard melanoma chemotherapy agent(145). No survival benefit, with this dose and schedule relative to dacarbazine, was observed in this trial. However, ipilimumab was more carefully evaluated at different doses and schedules, in addition to clinical management of the immune related toxicities using steroids and TNF blockers (146). Finally, in a randomized phase III three-arm clinical trial, metastatic melanoma patients received either: the gp100 vaccine, the gp100 vaccine plus ipilimumab, or ipilimumab alone. A survival benefit of 3.5 month was observed for patients in both groups receiving ipilimumab, compared to the group receiving the gp100 vaccine alone (147). As the first therapy to exhibit a survival benefit for patients with metastatic melanoma, ipilimumab was approved by the US Food and Drug Administration (FDA) for the treatment of advanced melanoma in 2010. Much more impressive than the mean survival benefit was the long term survival effect of ipilimumab. Compared with a long term survival of 5% of patients receiving the gp100 vaccine alone, 18% of the ipilimumab-treated patients survived beyond two years. This benefit of ongoing long term responses and survival well after completion of a relatively short course of therapy support the notion that T cell based immune therapies re-educate the immune system to keep tumours in check following therapy completion. Nonetheless, as with most oncology agents that provide benefit to a limited proportion of treated patients, much effort must be expended to define biomarkers predictive clinical responses.

Anti-PD1/Anti-PDL1

Programmed death 1 (PD1) is another co-inhibitory receptor expressed on activated T cells, B cells, natural killer cells and macrophages. The known two ligands for PD1 are PD1 ligand 1 (PDL1) and PDL2 expressed on a variety of immune cells. PDL1 and PDL2 are also highly

expressed on some tumour types, although they can also be upregulated on tumour cells by IFN- γ . Unlike CTLA4, PD1's major role is to limit the activity of T cells in peripheral tissues at times of an inflammatory response to infection and as well as to limit autoimmunity. PD1 therefore, predominantly regulates effector T cell functions within tumours and tissues, while CTLA4 predominantly regulates T cell activation. The binding of PD1 to PDL1/PDL2 results in the phosphorylation of two tyrosine residues in the intracellular tail of PD1(148). These tyrosine residues are part of an immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) and an Immunoreceptor Tyrosine-based Switch Motif (ITSM). ITSM then recruits either of two SH2-domain containing protein tyrosine phosphatases, tyrosine phosphatase 1 (SHP-1) and SHP-2. SHP-1 and SHP-2 then suppress activation of PI3K/Akt pathway(149). As a result, the expression of the pro-survival molecule Bcl-xL is decreased and expression of transcription factors associated with effector function including GATA-3, T-bet and Eomes are lost(149-151). The end result is diminished effector function in T cells characterized by decreased cytokine production, cytolytic function, and survival. PD1 is also highly expressed on Tregs, where it may enhance their proliferation in the presence of ligand in addition suppression of effector immune responses.

Generally, the PD1 pathway is thought to be mediate immune evasion in a process known as termed as "adaptive resistance"(131, 152). This stemmed from observations of PDL1 being absent from most normal tissues, whereas PDL1 can be induced by in practically any nucleated cell (153-156). Since hematopoietic cells are the main source of IFN- γ , it is reasonable to suggest that PDL1 expression could be induced as a result of cancer-induced inflammation. Tumour antigens can be presented to TILs by tumour cells, stromal cells as well as APCs. Following antigen stimulation TILs release IFN- γ leading to PDL1 induction in the tumour milieu. IFN- γ release can be a double edged sword. While IFN- γ enhances antigen processing and presentation as well as TIL differentiation, upregulation of PDL1 can also dampen T cell effector functions (157-159). Consequently, though the physiological role of PDL1 is to limit tissue damage via

dampening inflammation, PDL1 induction in the tumor microenvironment serves as a negative feedback mechanism to suppress T cell mediated antitumour immunity. This premise is supported by studies in melanoma showing a strong correlation between PDL1 expression in human melanocytic lesions and the presence of TILs. Laser-capture microdissection and quantitative real time PCR of tumour slides enabled the detection of IFN- γ at the interface of TILs and PDL1 expressing cells (157). Whereas IFN- γ remained undetectable within PDL1 negative tumour sections. Moreover, in murine tumour models, IFN- γ neutralization via administration of anti-IFN- γ antibodies abrogated PDL1 upregulation in the tumor microenvironment, demonstrating that IFN- γ is a major inducer of PDL1 *in vivo*(160). As such, PDL1 greatly contributes to the mechanisms of adaptive resistance shedding some light on immune evasion by cancer cells, despite endogenous tumour reactive T cells. Notably, cancer cells are also know to express PDL1 via IFN- γ independent mechanisms, as a small fraction of human cancers express high levels of PDL1 but lack TILs in the tumor microenvironment. There is now increasing evidence that tumour intrinsic oncogenic signaling may drive PDL1 expression. Several studies have revealed that aberrant EGFR, ALK and PI3K (via PTEN loss) signaling can directly upregulate PDL1 on cancer cells(160-162).

In NSCLC, renal cell carcinoma and metastatic melanoma, Nivolumab was the first monoclonal antibody (mAb) targeting PD1 to show significant clinical activity and is FDA approved for the treatment of both advanced melanoma and squamous NSCLC. In multiple melanoma clinical trials, Nivolumab has consistently induced objective response rates (ORR) of up to 40% in treated patients(163-166). Furthermore, in metastatic squamous NSCLC patients, Nivolumab also extended overall survival compared with standard chemotherapy. Moreover a recent phase I study evaluating the treatment of relapsed or refractory Hodgkin's lymphoma nivolumab produced an ORR as high as 87%(167). Nivolumab has also been used in combination therapy with ipilimumab. The overall survival of patients with advanced melanoma on a concurrent

regimen of nivolumab and ipilimumab was 94% after one year ([NCT01024231](#)). Pembrolizumab, another antagonistic mAb targeting PD1, has shown similar efficacy and safety compared with nivolumab in a phase I clinical trial in advanced melanoma (168, 169). It is now an FDA approved as a second line treatment for melanoma. In several clinical trials pembrolizumab has been effective in patients with advanced NSCLC, advanced gastric cancer, advanced bladder cancer, head and neck cancer, classical Hodgkin's lymphoma, and triple-negative breast cancer(152, 170-172). Monoclonal antibodies targeting PDL1 have also been developed and shown to be have therapeutic benefit in advanced human cancers. They include MDX-1105 (BMS) and MPDL3280A (Genetech/Roche), Pidilizumab (Medivation/ CureTech), MEDI4736 (AstraZeneca) and Avelumab (Merck-Sorono)(152). While PDL1 expression can to a degree be predictive of response to anti-PD1/PDL1 therapy, there are patients whose tumours express PDL1 but do not respond to these therapies and vice versa. This begs the need for continued investigation of these pathways as well rationale combinations with other therapies to provide better and longer lasting outcomes for cancer patients.

In summary, T cell mediated antitumour immune responses are regulated by delicate molecular interactions between T cells and the tumour microenvironment. A number of attractive negative regulatory mechanisms that limit the function of tumour reactive cells have been identified. Several prospective clinical trials based on these immunosuppressive mechanisms have been carried out. However, the expectations of these promising treatments have not translated into clinical success for all cancer patients suggesting that the list of tumour-associated negative immunoregulators is incomplete. Understanding these regulatory mechanisms is not only important from an academic viewpoint, but can also provide strategies for the development of new and/or improved immunotherapy treatments against cancer.

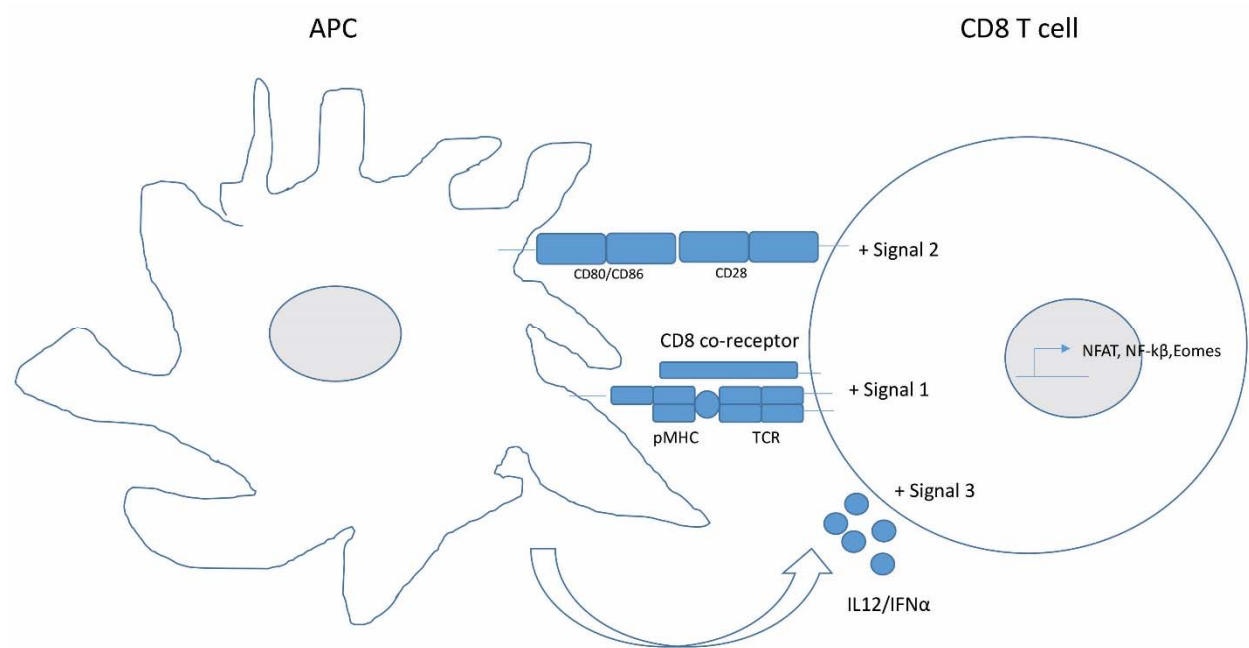


Figure 1: The three signal model of T cell activation

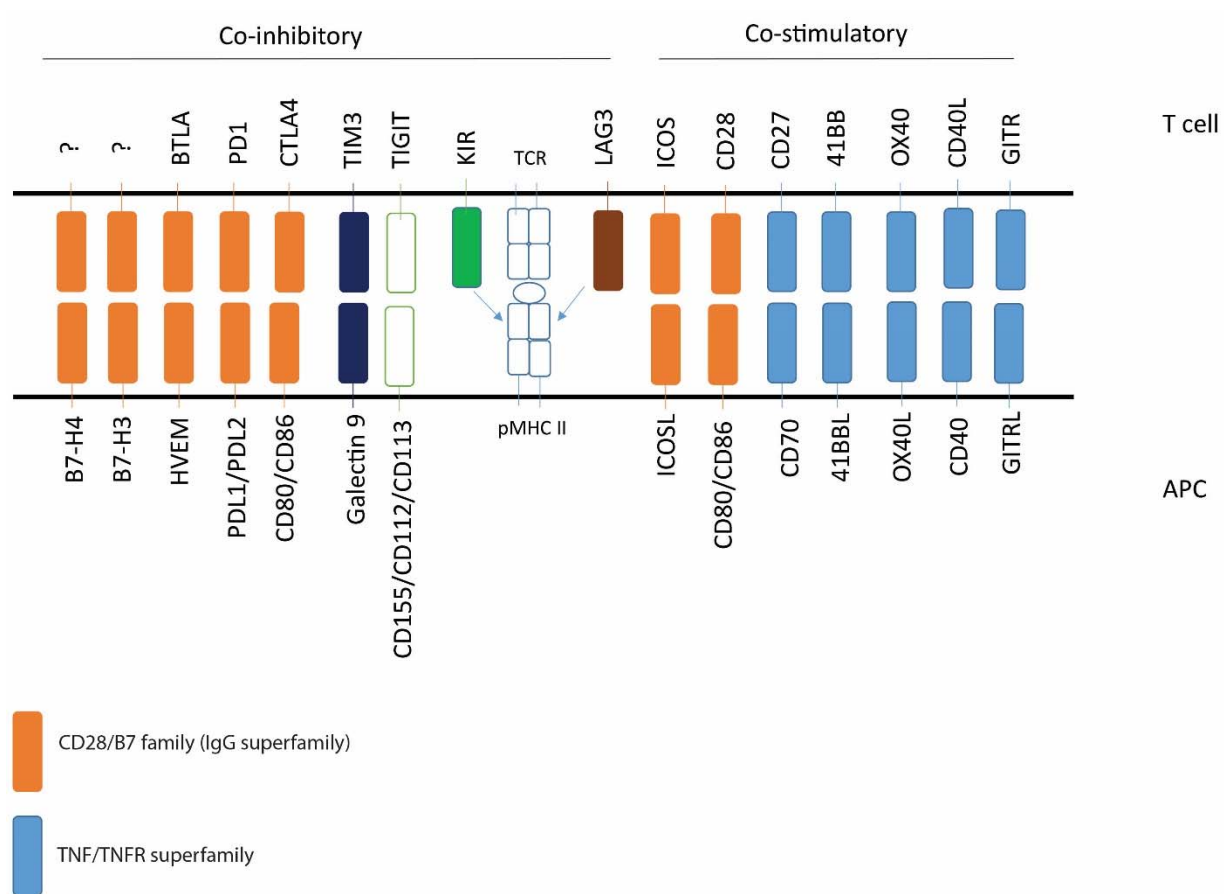


Figure 2: Co-stimulatory and co-inhibitory molecules and their respective ligands

Chapter 2

Enhancing T cell mediated anti-tumour immunity via HSP90 inhibition

Abstract

T cell-based immunotherapy has a major impact for cancer patients. However, the vast majority of patients do not benefit from these therapies. With increasing evidence that tumour intrinsic pathways modulate responses to immunotherapy, targeting these pathways provide a promising strategy for improving the efficacy of immunotherapy agents. Therefore, we made considerable efforts to investigate novel potential combinatorial targeted agents with immunotherapy using preclinical models. For this purpose, I used multiple paired patient-derived melanoma cell lines and their autologous TILs in a novel high-throughput chemical compound screen platform to identify compounds that improved the sensitivity of tumour cells to T cell killing. We hypothesized that these identified compounds could be used in combination with immunotherapy and improve the therapeutic antitumour outcomes when compared with the single-agent immunotherapy approach.

We successfully identified HSP90 inhibitors as agents that potentiate responses to T cell based immunotherapy by using this established platform to screen 850 compounds. *In vitro* studies we show that HSP90 inhibition synergistically improves T cell killing of multiple patient-derived human melanoma cell lines. Subsequently, we show that the clinically-relevant HSP90 inhibitor ganetespib potentiated responses to anti-CTLA4 and anti-PD1 immunotherapy in a preclinical murine tumour model. Mechanistically, upregulation of interferon response genes in the tumour cells, as a result of HSP90 inhibition, is essential for the enhanced T cell-mediated killing of tumour cells. These results not only highlight the therapeutic potential of HSP90 inhibition plus immune checkpoint blockade in cancer treatment, but also provide further supporting evidence of the essential role of interferon response genes in inducing death of tumour cells and their importance in potentiating response to T cell-mediated immunotherapy. Based on these

results, translation of the combination of HSP90 inhibitors and immunotherapy to the clinic is being actively pursued.

Tumour-intrinsic oncogenic pathways/proteins that interfere with response to T cell based immunotherapy

Over the years, the hallmarks of cancer described by Hanahan and Weinberg have been well supported and established by numerous studies. One of the original hallmarks of cancer described is the sustained proliferative ability of tumour cells driven by tumour intrinsic oncogenic signaling(173). There is now increasing evidence that tumour intrinsic oncogenic signaling not only promotes tumourigenesis but also contributes to immune evasion. It is no surprise therefore that some therapies targeting these oncogenic proteins have underlying immune mediated therapeutics effects and depletion of CD8 T cells results in abrogation of their therapeutic effect. In addition, inhibition of these tumour intrinsic signaling pathways can also lead to improved T cell responses including increased T cell trafficking and T cell mediated killing of tumour cells. In this section I will review some of the emerging tumour intrinsic oncogenic pathways that contribute to decreased or non-responsiveness to T cell mediated immunotherapy.

BRAF

BRAF is a component of the RAS-RAF-MEK-MAPK signal transduction pathway which is a critical regulator of cellular growth and survival. Till date, over 50 distinct activating point mutations have been identified the *BRAF* gene(174). The most common mutation described is a valine to glutamic acid substitution at amino acid position 600(V600E). It is by far the most frequent BRAF mutation, comprising more than 70% of BRAF mutations in melanoma (175, 176). *BRAF* is also mutated to a constitutively activated form in many other cancers types, including hairy cell leukemia, thyroid and colorectal (177). The wide expression of BRAF (V600E) mutation prompted the development

and clinical application of small-molecule–based pharmaceutical inhibitors selectively targeting BRAF (V600E) for the treatment of patients with metastatic melanoma (178). Although robust tumour regression is observed in more than half patients treated with the BRAF inhibitor vemurafenib, the development of resistance can sometimes occur (178, 179). As such, combination therapies with immune therapies is an attractive alternative to single agent treatment with multiple studies linking of MAPK activation with immunosuppression. For instance, studies in melanoma, have shown that the development of resistance to BRAF inhibitors is usually accompanied by upregulation of PDL1 on melanoma cells (180). In addition, oncogenic BRAF signaling in tumour cells results in the expression of immunosuppressive molecules such as VEGF, IL6 and IL10 in the tumour microenvironment and knockdown of BRAF significantly decreases secretion of these cytokines (181). *In vivo* studies also showed that inhibition of BRAF decreased VEGF production by tumour cells leading to an increase in the infiltration of T cells into the tumour(182). Finally, BRAF inhibition or blocking of MAPK signaling in melanoma cells augments antigen presentation, leading to improved recognition of treated cells by antigen specific T cells significantly (183-185).

Wnt/ β -catenin

The *Wnt* family of genes encodes 19 secreted glycoproteins which are ligands to receptor-mediated signaling pathways controlling cell fate and differentiation, cell proliferation, and cell motility(186). In canonical Wnt signaling, a Wnt protein binding to a member of the Frizzled (frz) transmembrane receptor family and initiates a Wnt signaling cascade. This leads to hyperphosphorylation of the Dishevelled protein (dsh) which then prevents the phosphorylation of β -catenin by the degradation complex (GSK-3, APC, and Axin)(187). Stable β -catenin then interacts with lymphoid enhancer factor/T cell factor (Lef/Tcf) and is translocated into the nucleus as a complex to stimulate target gene transcription. Noncanonical Wnt signaling, uses β -catenin-independent signaling mechanisms. The Wnt pathway has been implicated in multiple cancer

types. Particularly, gain of function mutations in β -catenin and loss of function mutations in negative regulators (such as TCF1, GSK-3, APC, and Axin) that lead to constitutive activation of Wnt/ β -catenin signaling(188) . In a recent melanoma study from Spranger et al, gene expression profiling was done on melanoma tumours that were classified as either T cell inflamed (had T cells infiltrates and associate genes) or non-T cell-inflamed(189). About 48% of non T cell inflamed tumours had aberrant activation of the Wnt/ β -catenin pathway. Follow up *in vivo* studies employed the use of spontaneous melanoma genetic models with stabilized β -catenin to decipher the importance of aberrant tumour-intrinsic β -catenin signaling in an anti-tumour immune response. In this model Spranger and colleagues found that these melanomas completely lacked tumour infiltrates. In addition, anti-PDL1 and anti-CLTA4 treatments had no therapeutic effect in this model. Finally, closer examination of these tumours showed a deficiency in CD103/CD8 α expressing DCs due to a defect in the production of the chemokine CCL4, which is critical in recruiting these cells. This study provides evidence of an oncogenic tumour intrinsic pathway that not only inhibits the generation of an anti-tumour response but also mediates resistance to immune checkpoint blockade therapy.

PI3K/PTEN/AKT

The Phosphoinositide 3-kinases (PI3K) pathway is an important pathway that generates phosphorylated intracellular lipids that affect a range of cellular activities including proliferation and survival. PI3K is regulated by the tumour suppressor gene PTEN which dephosphorylates activated lipids attenuating signaling(190). Over the years, several studies focused on inflammation-induced cancer progression have identified activating aberrations in PI3K signaling, either loss of function mutations in PTEN through or activating mutations in PIK3CA(191). The resulting suppressive tumour microenvironment was associated with an increase in immunosuppressive cytokines VEGFA, IL8, IL6, CSF1 accompanied by increased accumulation of tumour-associated macrophages. In addition, a recent study in melanoma by Peng and

colleagues also implicated PTEN loss in resistance to immunotherapy(192). Tumours with loss of PTEN protein expression had decreased infiltration of CD8 T cells in both BRAF/NRAS wild type and BRAF-mutant melanomas. Furthermore, analysis of samples collected from treated patients revealed that low PTEN expression is associated with reduced LCK expression, Lscore, and cytolytic activity in melanomas, consistent with decreased immune infiltration with PTEN loss. Moreover, PTEN deletions and loss of function mutations were also considerably enriched in the non-T cell–inflamed tumours in the melanoma TCGA samples. In anti-PD1 treated patients PTEN loss was found to be enriched in non-responders compared to responders. In addition, decreased T cell infiltration into tumours and PTEN loss also contributed to decreased T cell mediated lysis of tumours(193). Notably, these analyses of the melanoma TCGA provided support that loss of PTEN in melanomas is largely mutually exclusive with β -catenin pathway alterations. These provide evidence that multiple distinct genetic events can give rise to immune exclusion. Nevertheless, oncogenic activation of the Wnt/ β -catenin and PI3K pathways do not account for all of the tumours with the non-T cell–inflamed phenotype, suggesting that additional pathways exist that contribute to immune exclusion warranting further exploration.

HSP90

HSP90 biology

Heat shock protein 90 (HSP90) is a molecular chaperone whose role is to properly fold over 200 client proteins thereby maturing, stabilizing, regulating and activating their function. Higher eukaryotes possess several isoforms of HSP90 located in the cytoplasm, mitochondria and endoplasmic reticulum. HSP90 α , inducible under stressful conditions, and HSP90 β , which is constitutively expressed, are the two major cytoplasmic isoforms. While, GRP96 is expressed in the endoplasmic reticulum and TRAP-1 in the mitochondrial matrix (194-196). HSP90 structurally consists of a homodimer, with each protomer having three functional domains: An ATP binding N-terminal domain (N-domain), a middle domain (M-domain) and a dimerizing C-terminal domain

(C-domain) (Figure3)(197). All three domains provide co-chaperon and client protein binding sites. Interaction of HSP90 with its plethora of client proteins is facilitated by co-chaperons that serve as adaptor molecules providing selectivity and specificity for client proteins. Over 20 co-chaperons have been identified to date and in addition to recruiting specific client proteins, they activate or inhibit the HSP90 ATPase. Usually, different co-chaperons work together to enable client protein maturation. Thus, co-chaperon complexes are to a certain extent dictated by specific client proteins. All-in-all the proper folding of a client protein requires assembly of an HSP90 complex consisting of the client protein, co-chaperons and HSP90(198).

The HSP90 chaperone cycle is a tightly regulated process including multiple steps (Figure 4). It begins with the assembly of an 'early complex' involving association of a client protein with an HSP70/HSP40 complex. The co-chaperon Hop then facilitates interaction of the early complex with HSP90 and transfer of the client protein to HSP90 to form an 'intermediate complex'. Depending on the client protein additional co-chaperons can be recruited to facilitate this transfer. ATP binding recruits additional co-chaperons that stabilize the HSP90 complex and promote release of the Hop and HSP70 from the complex. ATP hydrolysis induces conformational changes that enable folding of the client protein and leads to release of folded protein ADP and co-chaperons completing the cycle(198).

Over the years, a broad spectrum of over 200 HSP90 client proteins have been identified. These encompass many protein classes including steroid hormones, protein kinases, transcription factors, RNA polymerases, PI3 kinase-like kinases and NLR receptors, amongst others (199-201). Many known oncoproteins such as BRAF, HER2, EGFR, BCR-ABL and MET are client proteins of HSP90, thereby affirming the importance of HSP90 in tumour growth, survival, invasion and metastasis (Figure 5). It is thought that the instability of these proteins caused by mutations, coupled with environmental stress and genetic instability, produce a great dependency of tumour cells on HSP90. Therefore, the number of HSP90 complexes found in

tumour cells are up to 10 fold higher than those found in normal cells. This phenomenon has attracted the use of HSP90 inhibitors in cancer treatment, as inhibition of HSP90 promotes concurrent inhibition of multiple oncogenic pathways.

HSP90 inhibitors in cancer treatment

The first two HSP90 inhibitors developed were the two natural products, geldanamycin (GM) and radicicol (RD)(202, 203). Both compounds were found to inhibit HSP90 via competitive binding with ATP to its N-terminal regulatory pocket. However, these two compounds were not clinically relevant given their poor stability and toxicities *in vivo*, stemming from their reactive chemical structures. Nevertheless, these molecules served as pathfinder and tools for better understanding HSP90 biology in tumours. They also provided valuable pharmacophores for next-generation inhibitors (Figure 6). The first generation HSP90 inhibitor compounds were derivatives of the antibiotic, GM, created by substituting the non-essential C-17 methoxy group on the bezoquinone moiety with various amines. 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG) was the first of its kind to enter clinical trials (204). While 17-AAG (tanespimycin) retained the important biological features of GM, it had an improved toxicity profile (205). Various dosing schedules and formulations, as a single agent therapy, were explored in divers Phase I clinical trials with the most promising clinical activity observed being stable disease (SD). In Phase II trials in melanoma, prostate and renal cell cancer, no objective responses were noted with single agent tanespimycin despite the presence of known target client proteins in these cancers (206, 207). On the other hand, in a Phase II trial of tanespimycin in combination with trastuzumab in HER2+ metastatic breast cancer (MBC) patients progressing on prior trastuzumab, an overall response rate (ORR) and clinical benefit rate (CBR) of , 22% and 59%, respectively was observed(208). The median progression free survival (PFS) and overall survival (OS) were 6 and 17 months, respectively. This indicated that efficacy could be obtained in a combination setting as well as

with patient enrichment (HER2 is a client protein sensitive to the HSP90 inhibitor). Nevertheless, due to poor a pharmaceutical and toxicity profile, 17-AAG failed to advance further.

17- desmethoxy-17-N,N-dimethylaminoethylaminogeldanamycin (17-DMAG) developed by Kosan Biosciences overcame the solubility issues observed with 17-AAG (203). Instead of the methoxy group at C-17, 17-DMAG contains the ionizable N,N-dimethylethylamine group which provided the much-needed improvement in water solubility, oral bioavailability and equal, if not greater, antitumour activity than 17-AAG. In 2004, 17-DMAG (alvespimycin) was investigated in clinical trials both as an oral and intravenous agent (207, 209-211). In patients with castrate-resistant prostate cancer (CRPC), acute myeloid leukemia, clear cell renal cancer and metastatic melanoma objective responses, including some CRs, were observed (210-212). In addition, SD greater than 6 months were also reported in 3 patients with chondrosarcoma, CRPC and clear cell renal cancer. Toxicities such as diarrhea, fatigue, nausea, liver, lung, ocular and cardiac toxicities reported in these trials. Comparable to tanespimycin, a phase I clinical trial indicated that alvespimycin may be most beneficial in combination with trastuzumab. In this trial a PR in a HER2+ MBC patient and SD lasting longer than 6 months in 6 of the 28 other patients was observed with the combination of alvespimycin and trastuzumab(203). However, the clinical development of alvespimycin was halted in 2008 by Kosan due to strategic reasons, despite these encouraging objective responses. However, alvespimycin is currently under evaluation by the National Cancer Institute for the treatment of patients with relapsed small lymphocytic lymphoma, B-cell prolymphocytic leukemia and chronic lymphocytic leukemia (CLL)(203).

In summary, research efforts by both academia and industry to develop newer HSP90 inhibitors with better bioavailability and toxicity profiles to provide increased therapeutic benefit have been fueled by GM derivatives. The pursuit for these much-improved small synthetic molecules resulted in the development of better HSP90 inhibitors known as second generation HSP90 inhibitors. Studies with the first generation HSP90 inhibitors also informed what tumour

subtypes more likely to benefit from HSP90 inhibitors facilitating the clinical path of upcoming agents.

Ganetespib: a second generation HSP90 inhibitor

Ganetespib (STA-9090) a second generation HSP90 inhibitor with a prevalent triazolone moiety, developed by Synta pharmaceuticals. This inhibitor has a considerably smaller unique scaffold that distinguishes it from the first generation GM analogues (213). As reviewed above, the chaperone activity of HSP90 is associated with ATP-driven conformational changes within the N-terminal domain. Due to their larger sizes, the GM analogues can only occupy the ATP-binding pocket in the open conformation, whereas, ganetespib is able to enter the ATP-binding pocket in the closed conformation due to its smaller size (214). The lack of binding restriction to the ATP pocket contributes greatly to the higher *in vitro* potency of ganetespib compared to the GM analogues. Till date, ganetespib has been or is currently being tested in over 25 clinical trials in both hematologic and solid tumours. The dosing schedule for ganetespib was evaluated for solid malignancies in two clinical trials: it was either given intravenously weekly for 3 weeks in a 28-day cycle or twice-weekly dosing for 3 weeks in a 28-day cycle. Ganetespib was well tolerated in patients with mostly grade 1 or 2 toxicities that were easily manageable. Following these studies, 200 mg/m² was the recommended Phase II dose for the weekly dosing. One patient with advanced melanoma achieved a PR while 2 with non-small cell lung cancer (NSCLC) achieved SD (202, 203). Ganetespib was also evaluated in two Phase I single agent targeting hematologic malignancies The recommended doses for further study in this population were 200 mg/m² once weekly and 90 mg/m² twice weekly. The most common adverse events in both these trials were mild-to-moderate diarrhea and fatigue that were reversible and easily manageable. Finally, ganetespib was also evaluated in Phase I trials in patients with NSCLC, GIST and MBC. As a single agent in advanced NSCLC, ganetespib showed encouraging activity in patients whose tumours harboured an EML4-ALK rearrangement, were naïve for crizotinib treatment and were

either a non-mutant EGFR or KRAS. Amongst eight such patients evaluated, there were four PRs and a median PFS of 8.1 months. This provided a foundation for a Phase II study of ganetespib monotherapy in patients with ALK-positive disease who were crizotinib-naïve. Supporting data from preclinical studies showing synergistic antitumour effects provided rationale for combining HSP90 inhibitors with taxanes. As such a randomized Phase II GALAXY-I trial combining ganetespib with docetaxel, an extended OS was observed with ganetespib and docetaxel compared to docetaxel alone in the second-line setting in patients with advanced NSCLC. This trial encouraged a phase III trial of this combination in NSCLC known as the GALAXY-2 trial and results are currently awaited (202, 203).

Results

HSP90 inhibition enhances T cell mediated killing of melanoma cells

To identify compounds that increase the sensitivity of human melanoma cells to T cell mediated killing, we utilized paired patient derived human melanoma cell lines and their autologous tumour infiltrating T cells (TILs), derived from our active adoptive cell therapy program, in a high throughput *in vitro* screen of 850 bioactive compounds. Two human melanoma cell lines 2549 (wild type for *BRAF*, *NRAS* and *cKIT*) and 2338 (*BRAF* V600E mutated) were treated with 1uM of each compound for 24 hrs or DMSO as a control. The treated tumour cells were then washed and incubated with autologous TILs for 3hrs at a predetermined ratio, and the levels of cleaved caspase 3 assessed as a readout of apoptosis. To quantify the interactive effect of the compounds on T cell mediated killing, a comboscore was calculated from the percentage of T cell-induced apoptosis in tumour cells with or without compound treatment. Compounds that enhance the sensitivity of tumour cells to T cell mediated killing have comboscores >1. Amongst the top candidates that increased the sensitivity of treated tumour cells to T cell killing were all three HSP90 inhibitors in the screen: 17-DMAG, BIIB021 and 17-AAG (Figure 7A and Supplemental Figure 1A). To validate these findings, we utilized a newer generation HSP90 inhibitor, ganetespib, which has been reported to exhibit greater potency in preclinical tumour models and reduced toxicity in rodents compared to other 1st and 2nd generation HSP90 inhibitors. Additionally, ganetespib also has a comparably better safety profile in patients (202, 215). Confirming the screen results, varying concentrations of ganetespib increased the sensitivity of 2549 and 2338, and additional human melanoma cell lines 2400 and 2559 (*BRAF* V600E mutated), to T cell mediated killing (Figure 7B and Supplemental Figure 1B). Furthermore, the combination of ganetespib and TIL treatment was formally synergistic in tumour cell killing, especially at lower concentrations of ganetespib, as indicated by combination indexes (CI) < 1 (Figure 7C and Supplemental Figure 1C) calculated by the Chou-Talalay method(216). Taken

together, these data suggest HSP90 inhibitors as a combination partner to improve response to immunotherapy.

The *IFIT* genes are essential for the enhanced T cell killing of tumour cells following HSP90 inhibition

To mechanistically understand how HSP90 inhibition increased sensitivity of tumour cells to T cell killing, we performed gene expression analysis of the human melanoma cell lines 2400, 2338, 2549 and 2559 treated with either DMSO, as a control, or ganetespib alone. Two commonly used bioinformatics tools, gene set enrichment analysis (GSEA) and Ingenuity Pathway Analysis (IPA), both implicated interferon response genes as being significantly upregulated following treatment with ganetespib, with interferon signaling being the highest-scoring canonical pathway by IPA (Supplemental Figure 2A-C and Figure 8A). Upregulation of interferon response genes in multiple melanoma cell lines by ganetespib was confirmed by quantitative real time PCR and Western blot analyses, most strongly for members of the IFN-induced protein with tetratricopeptide repeats (*IFIT*) gene family: *IFIT1*, *IFIT2* and *IFIT3* (Figure 8B-C & Supplemental Figure 4A-B). Furthermore, the *IFIT* genes were upregulated *in vivo* following treatment with ganetespib (Supplemental Figure 3). To determine the importance of the *IFIT* genes in the synergy observed between ganetespib treatment and T cells in tumour cell killing, *IFIT* gene expression was perturbed in tumour cells. *IFIT*-silenced and control tumour cell lines were generated by simultaneously transducing tumour cells with *IFIT1*, *IFIT2* and *IFIT3* small hairpin RNAs (shRNA) or with scrambled shRNA, respectively (Figure 9A and Supplemental figure 5A). DMSO treated control and *IFIT*-silenced cell lines were equally sensitive to T cell killing. However, silencing the *IFIT* genes abrogated the enhanced killing of the melanoma cells potentiated by ganetespib (Figure 9B and Supplemental Figure 5B). Conversely, overexpressing *IFIT1*, *IFIT2* and *IFIT3* in human melanoma cell lines enhanced the sensitivity of human melanoma cell lines to T cell killing, thereby recapitulating the effects of ganetespib treatment (Figure 9C-D and

Supplemental Figure 5C-D). Interrogating apoptotic molecules upstream of caspase 3 revealed a dramatic decrease in BCL2 expression following overexpression of the *IFIT* genes (Figure 9E & Supplemental Figure 5E), suggesting that the *IFIT* genes promote sensitivity to apoptosis of the tumour cells. Taken together, our studies indicate that upregulation of *IFIT* genes is essential for the enhanced T cell killing of melanoma cells following HSP90 inhibition.

HSP90 inhibition potentiates responses to anti-CTLA4 *in vivo*

We next assessed whether HSP90 inhibition could enhance responses to T cell directed immunotherapy *in vivo*. MC38/gp100 tumour bearing mice were treated with solvent/antibody control (vehicle), ganetespib, anti-CTLA4 or the combination of ganetespib and anti-CTLA4. Treatment with ganetespib was started at the same time as anti-CTLA4 (Figure 10A). The combination of ganetespib and anti-CTLA4 conferred a better anti-tumour response, compared to either treatment alone (Figure 10B) and a better survival compared to either treatment alone (Figure 10C) with the median survival in the vehicle = 21days, ganetespib = 24 days, anti-CTLA4 = 22.5 days and the combination of ganetespib and anti-CTLA4 = 28 days. Taken together, the results of the *in vivo* studies indicate that HSP90 inhibition enhances responses to anti-CTLA4 therapy.

The combination of HSP90 inhibition and anti-CTLA4 treatment enhances the effector function of CD8 T cells

To investigate whether HSP90 inhibition and anti-CTLA4 treatments modulate the immune cell population infiltrating the tumour, C57BL/6 mice were treated with vehicle, ganetespib, anti-CTLA4, or the combination of ganetespib and anti-CTLA4, starting day 7 after inoculation with MC38/gp100 tumours (Figure 11A). In tumours harvested on day 18 after tumour inoculation, the absolute number of CD8 T cells (Figure 10B) and Ki67 positive CD8 T cells (Supplemental Figure 6A) were highest in the combination treatment, but the difference between the anti-CTLA4 only

and combination groups was not statistically significant, suggesting that ganetespib treatment does not further increase trafficking or proliferation of CD8 T cells in the tumour. However, the number of T regulatory cells (Tregs) was significantly decreased in the treatment groups compared to the vehicle (Figure 11C); both anti-CTLA4 and HSP90 inhibitor treatments alone have been shown to decrease the number of tumour-infiltrating Tregs (217, 218). This resulted in an increased CD8 T cell to Treg ratio in the treatment groups, with the difference between the combination group and the other groups being statistically significant (Figure 11D). Other cell populations such as myeloid derived suppressor cells (MDSCs) and effector CD4 T cells remained unchanged (Supplemental Figure 6B-C). To investigate whether the increase in CD8 T cell to Treg ratio had an impact on CD8 T cell effector function, production of the cytotoxic molecules granzyme A and granzyme B by CD8 T cells was assessed. The number of CD8 T cells making granzymes A and B was significantly higher in the combination treatment group compared to the other treatment groups (Figure 11E-F & Supplemental Figure 6D), suggesting that the combination treatment enhanced the effector function of CD8 T cells infiltrating the tumour. Furthermore, to interrogate whether the generation of antigen specific cells was affected by treatment, splenocytes isolated from the treated mice were stimulated *ex vivo* with either DMSO as a control, gp100 or p15E peptides (MHC I restricted peptides expressed by the MC38/gp100 tumour), and IFN-gamma production was assessed by ELISPOT. Results indicated a significant increase in IFN-gamma producing cells following stimulation with gp100 peptide in the treatments groups with the increase being the most dramatic in the combination treatment group (Figure 12), suggesting that the treatment of ganetespib and anti-CTLA4 enhances antigen specific CD8 T cell generation. Stimulation with P15E peptide showed a similar trend although the increase was not statistically significant in the anti-CTLA4 and combination groups. Taken together, these results suggest that the combination of HSP90 inhibition and anti-CTLA4 therapy enhances the generation of antigen specific CD8 T cells and the effector function of CD8 T cells.

Discussion and future directions

In this study, HSP90 inhibitors were identified as promising combination partners with immunotherapy. Many known oncogeneic drivers are client proteins of HSP90 which regulate tumor intrinsic pathways, some of which may provide a route of interference with response to immunotherapy (203, 214, 219, 220). However, HSP90 inhibitors as single agents, have shown limited therapeutic activity in cancer patients but encouraging responses when combined with other targeted therapies (207, 210). Studies from our group and others provide an indication for combining HSP90 inhibition with immunotherapy. Preclinical studies from the Storkus group using the first generation HSP90 inhibitor 17-DMAG provided initial evidence of HSP90 as a tumor intrinsic molecule that could be targeted to enhance responses to immunotherapy in a vaccine model (221, 222). We now provide evidence that HSP90 inhibition enhances T cell mediated killing of melanoma cells and, for the first time, potentiates responses to immune checkpoint blockade *in vivo* (Supplemental figure 5).

HSP90 is ubiquitously expressed and is vital in a number of important processes involved in the integration of innate and adaptive immune responses. These include antigen processing and presentation, activation and cross-priming of lymphocytes, and DC activation/maturation. Therefore, combination therapy with inhibition of HSP90 does not initially appear a reasonable approach for improving the T cell mediated antitumor functions observed with immune checkpoint blockade. As part of the classical endogenous pathway of antigen processing and presentation, peptides are delivered to the surface bound to MHC I molecules(219). Data from *in vitro* studies suggest that HSP90 may be vital for the binding and delivery of chaperoned, antigenic peptides with MHC I molecules to the cell surface. Moreover, via a nonclassical, MHC II– mediated mechanism for presentation of intracellular tumor antigens HSP90 can also facilitate direct tumor recognition by CD4 T cells . While, these studies provided valuable information on the role of HSP90 in antigen processing and presentation, it should be noted that the model systems used involved either a complete knockout of HSP90 or the use of extremely high concentrations of the

first generation inhibitors (up to 30uM). Studies from our group and other groups show that inhibiting HSP90 transiently with lower concentrations of the HSP90 inhibitors have provided evidence that HSP90 inhibition can enhance responses to immunotherapy. For instance, 17-AAG has been shown to upregulate the expression of melanoma antigens on the surface thereby enhancing T cell recognition. This observation was also validated and confirmed in an expanded study evaluating the capacity of 12 different and structurally diverse HSP90 inhibitor compounds, to increase the expression of tumor-specific differentiation antigens in a panel of glioma and melanoma cell lines. In addition, HSP90 inhibition has also been shown to upregulate MHC I expression, a synergistic effect that combines with the enhanced antigen expression to promote increased T cell recognition of treated tumours. Moreover, studies from the Storkus' group, using cells expressing the well-established HSP90 client protein EphA2, provide compelling evidence of HSP90 inhibition enhancing response to immunotherapy in a vaccine model(221, 222). In these series of studies, 17-DMAG enhanced tumour recognition by EphA2 specific CD8 T cells via enhanced proteasomal degradation of and subsequent MHC-dependent presentation of EphA2 derived antigens. In complementary *in vivo* studies, EphA2-expressing sarcoma tumours were treated with 17-DMAG and an EphA2 vaccine. The stimulation of adoptively transferred T cells by the EphA2 vaccine resulted in the generation CD8 T cells which potentiated the tumour inhibitory effects of 17-DMAG. In addition, 17-DMAG treatment led to a reduction in suppressor cell populations, including Tregs and MDSCs. This is consistent with our current study, where we see an enhancement of T cell mediated killing of ganetespib treated tumour cells by T cells *in vitro*. As well as, a potentiation of immune checkpoint blockade function *in vivo*, characterized by delayed tumour progression, enhance CD8 T cell function and a decrease in Tregs. The decrease in Tregs is probably due to the fact that Tregs are extremely dependent on the HSP90 homologue GRP94 to maintain TGF- β production, as recently described in a study from Zhang and colleagues(223). However, follow up studies will be need to confirm this phenomenon in this setting. Taken together, the previously considered negative characteristic of HSP90 inhibitors

could actually to assist in the generation of antitumour immunity and potentiate various forms of immunotherapy.

Another setting in which HSP90 inhibitors have been reportedly used is in inflammatory autoimmune models. A high titer of autoantibodies to HSP90 in the sera of systemic lupus erythematosus (SLE) patients, as well as the elevated expression of HSP90 in kidneys and sera of SLE patients, prompted the preclinical investigation of HSP90 inhibition in SLE. In a study by Shimp and colleagues, the treatment of MRL/lpr mice (the most commonly used mouse model of SLE) with the HSP90 inhibitor 17-DMAG led to a decrease in the number of follicular B cells accompanied by a decrease in the levels of double negative T cells and the CD4/CD8 ratio. This provided evidence that HSP90 may be important in regulating T cell differentiation and activation and blocking these events through HSP90 inhibition may be a promising therapeutic strategy in lupus (224-226). In another study, DCs were shown promote autoimmune responses in SLE. GRP94 was shown to induce DC activation in SLE, as such blocking the maturation of these cells via HSP90 inhibitor treatments was proposed as a potential therapeutic approach (227, 228). These studies provide evidence that HSP90 is important in various aspects of the generation of an immune response. Notably, the impairment of the generation and functions of different immune cell compartments would come as a result of frequent and/or continuous dosing of the HSP90 inhibitor. For instance, administration of the HSP90 inhibitor on a schedule of twice a week for 14 weeks in a murine model of SLE or a schedule of once daily for 14 weeks in an epidermolysis bullosa acquisita model (224, 229). However, in the oncology setting, studies have shown tumour selectivity and retention of HSP90 inhibitors. These properties of HSP90 inhibitor compounds have permitted intermittent dosing regimens that lead to effective and sustained inhibition of chaperone inhibitory activity within the tumour compartment coupled with restricted systemic immune system drug exposures. Therefore there has to be careful consideration for length of the duration of HSP90 inhibitor treatments in clinical evaluation.

We also demonstrate for the first time that HSP90 inhibition upregulates interferon response genes in the tumor, notably *IFIT1*, *IFIT2* and *IFIT3*, both *in vitro* and *in vivo*. There is now increasing evidence that The *IFIT* genes, whose major roles have been previously described in response to viral infections, also have a role in tumour biology. Particularly, *IFIT2* overexpression in tumour cells promotes tumour cell death(230). In addition recent studies reveal that a higher expression of interferon response genes, including the genes interrogated in this study, have been associated with long term benefits to anti-CTLA4 immunotherapy across multiple cancer types(231). In this study by Chiappinelli and colleagues, DNA methyltransferase inhibitors (DNMTis) triggered cytosolic sensing of double-stranded RNA (dsRNA) resulting in the increased expression of type I interferon response genes and apoptosis of ovarian cancer (OC) cells. Basal levels of these interferon response genes positively correlated significantly with an endogenous retrovirus (ERV) signature for multiple tumor types from samples in The Cancer Genome Atlas. We now show specifically that *IFIT2* and other family members *IFIT1* and *IFIT3*, when overexpressed in tumor cells, enhance T cell killing of tumor cells. Importantly, silencing of these genes abrogated the enhanced T cell killing of melanoma cells following HSP90 inhibition. This provides additional evidence that these genes play an essential role in inducing death of tumor cells and their importance in potentiating response to T cell mediated immunotherapy. The mechanism of interferon response genes upregulation following HSP90 inhibition in our melanoma cell lines is still unknown. Evaluation of the presence of increased ERV expression following HSP90 inhibition proved futile (data not shown). This was probably due to the fact that our HSP90 inhibitor treatments were only 24hrs long, which is not enough time to reverse any existent DNA hyper methylation, suggesting an alternate mechanism. It is known that HSP90 inhibition leads to the upregulation of the heat shock protein family member HSP70. In addition, HSP70 has been shown to be upregulated and secreted in situations of cellular stress, such a viral infection or heat shock. Secreted HSP70 acts as a PAMP, and can activate DCs via TLR2/TLR4 inducing a type 1 interferon response as well as induction of antigen

presentation(232). It is possible that the stress induced by HSP90 inhibition leads to the secretion of HSP70 which can in turn induce an interferon response in our treated melanoma cell lines. Examination of gene expression data shows an upregulation of HSP70 in ganetespib treated cell lines (Supplemental figure 6E). In addition, mass spectrometry analysis of supernatants obtained from ganetespib treated cells indicate an increase in HSP70 secretion compared to control (Supplemental figure 6F). However, studies to confirm the secretion and stimulation of tumour cells by HSP70 following HSP90 inhibitor will have to be performed.

In summary, this study provides evidence that HSP90 inhibition can potentiate T cell mediated anti-tumor immune responses and supports exploration of the combination of immunotherapy and HSP90 inhibitors in the clinic. Moreover, with the recent identification of aberrations in the IFN pathway as a mechanism of immune resistance, this provides another avenue of investigation of whether HSP90 inhibitors may play a role in reversing this resistance (233-235).

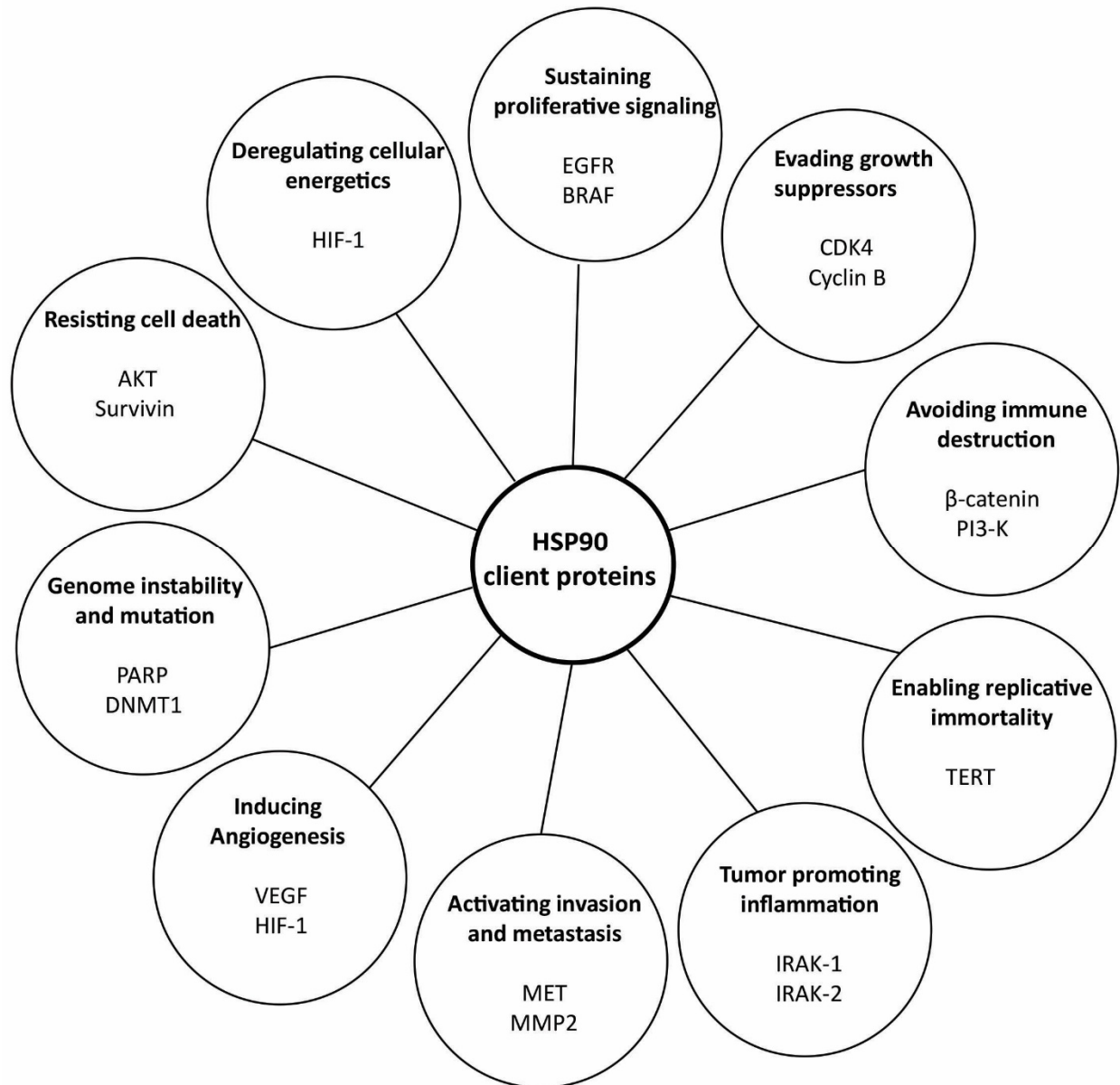


Figure 3: Several HSP90 clients involved in the hallmarks of cancer

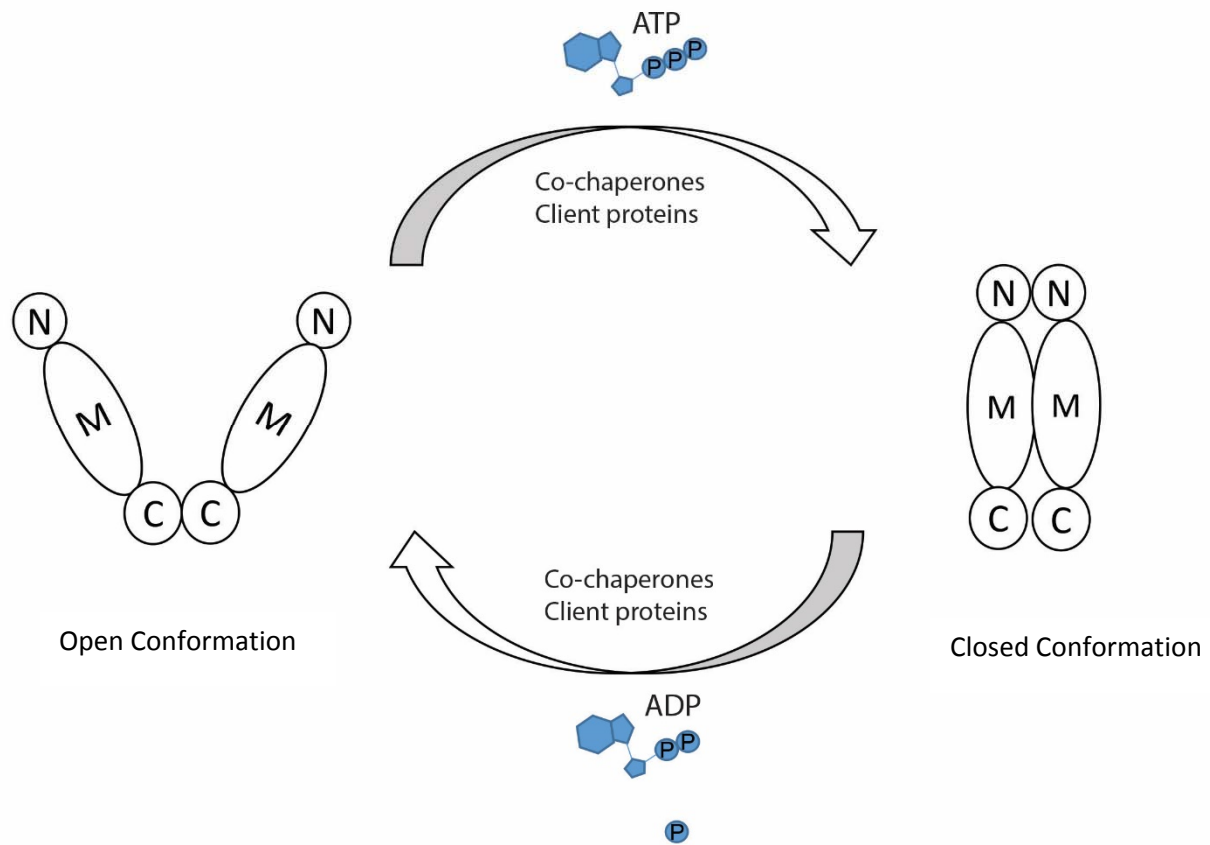


Figure 4: HSP90 structural domains and conformations

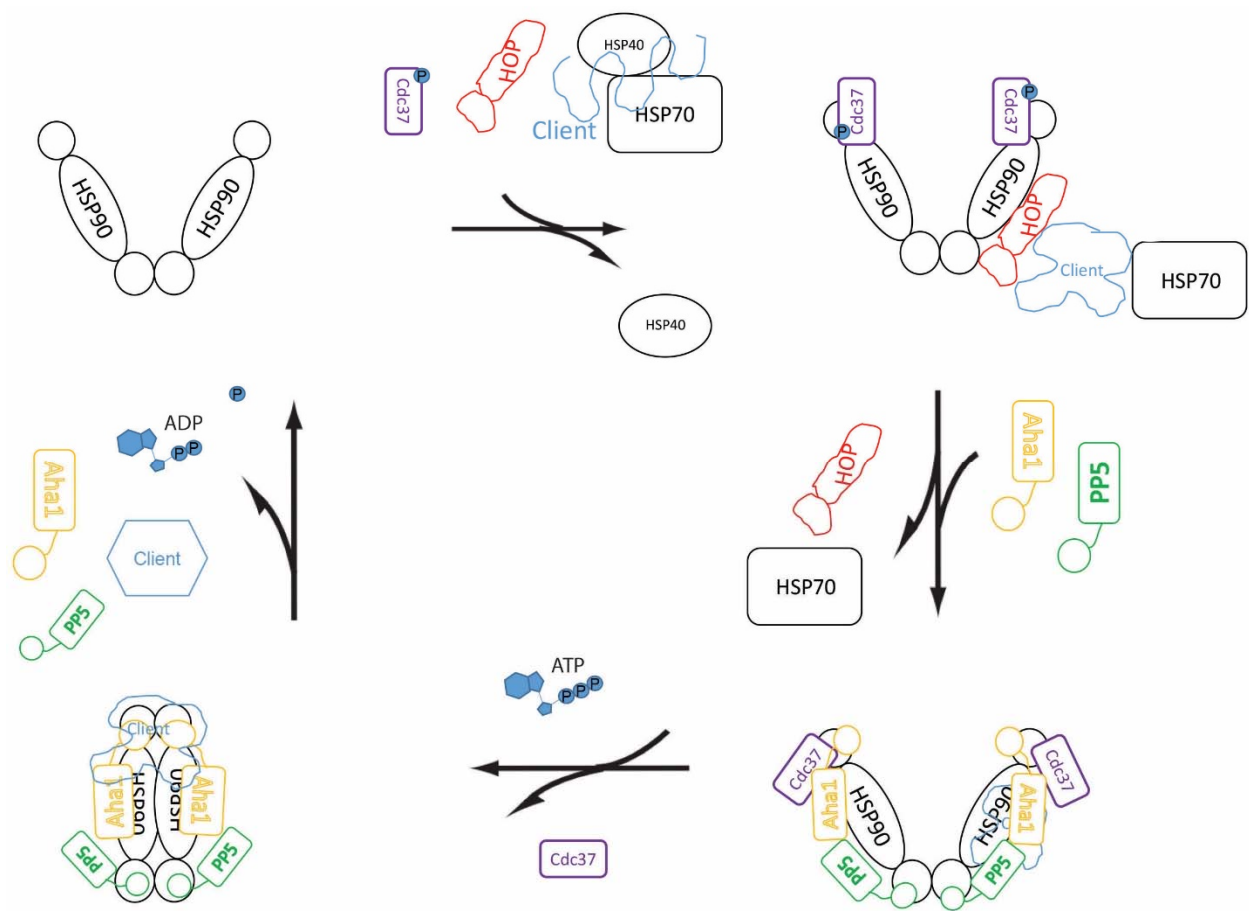
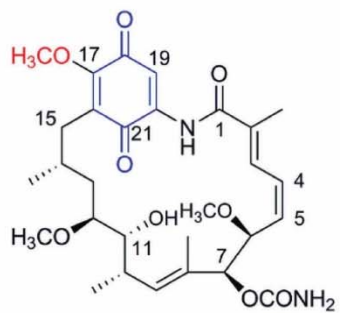
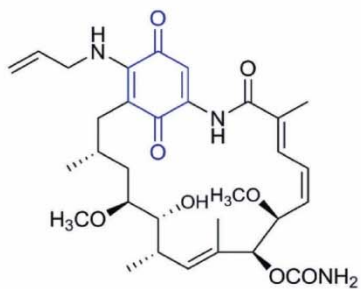


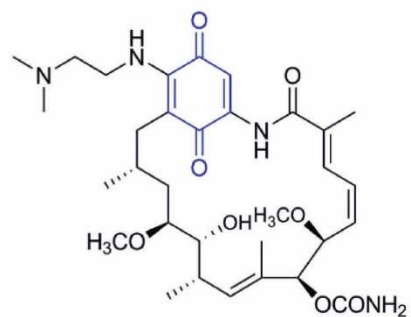
Figure 5: The HSP90 chaperone cycle



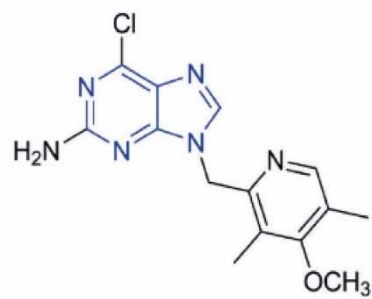
Geldanamycin



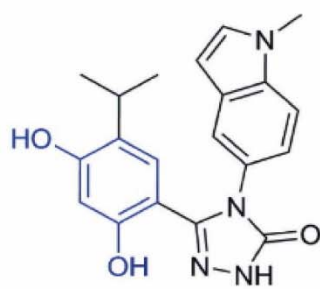
17-AAG



17-DMAG



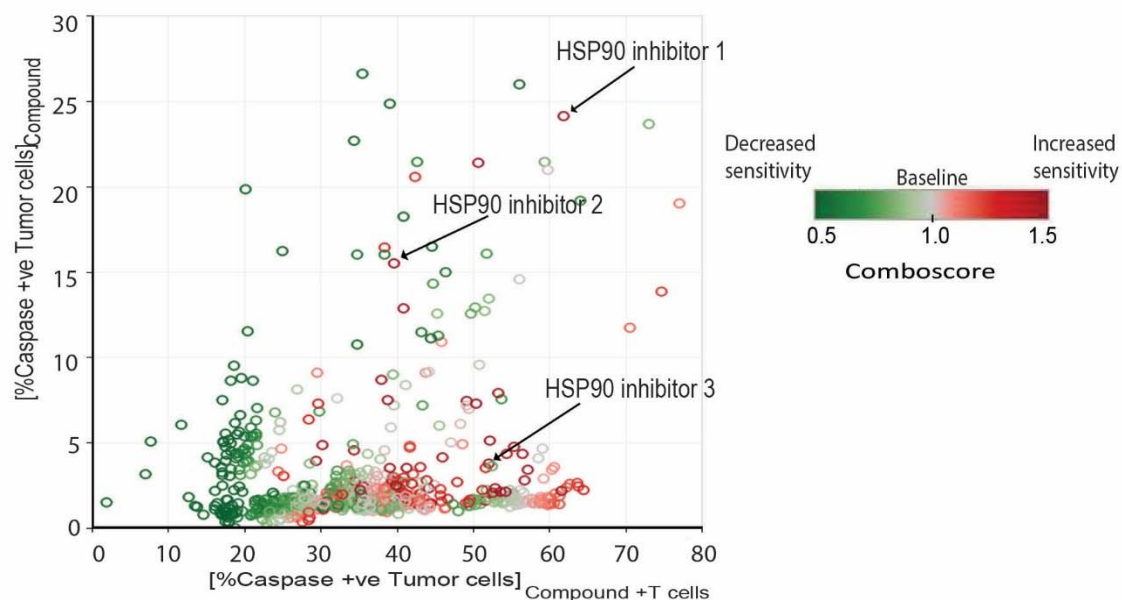
BIIB021



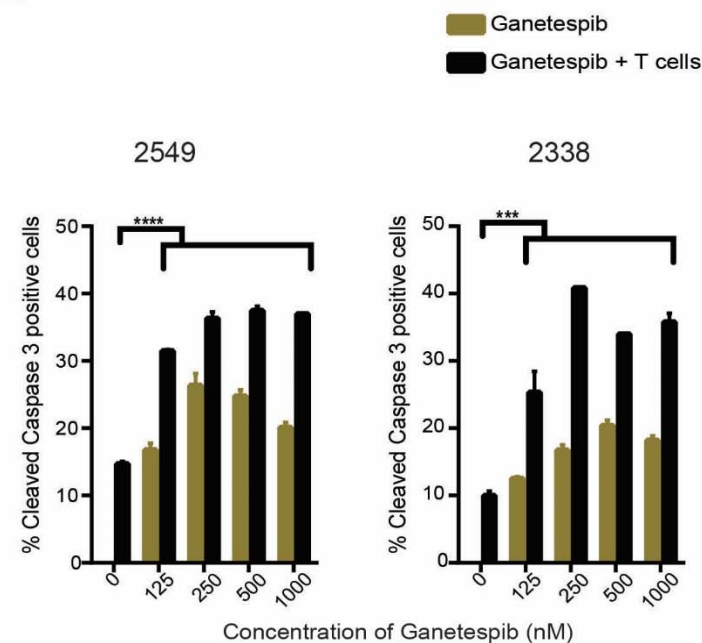
Ganetespib

Figure 6: Examples of first and second generation HSP90 inhibitors

A



B



C

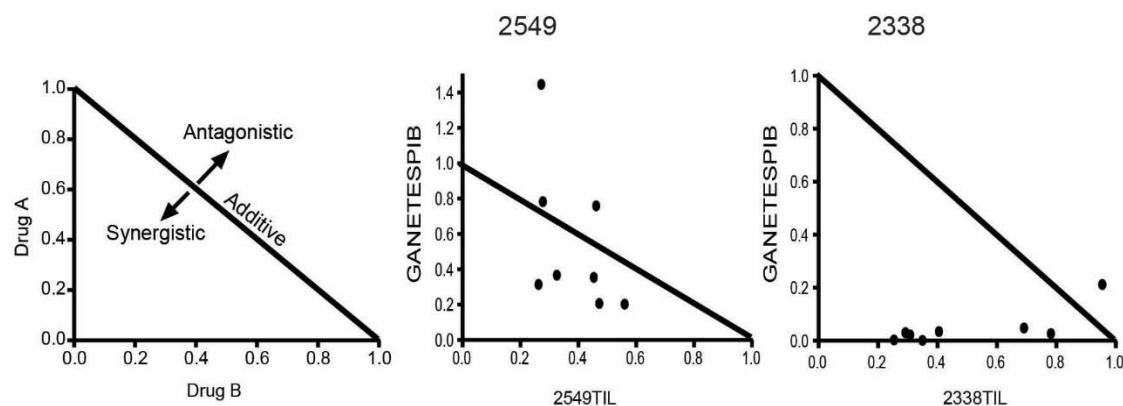
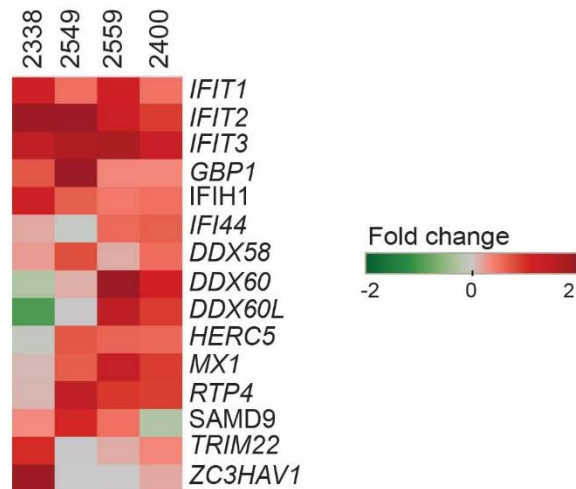


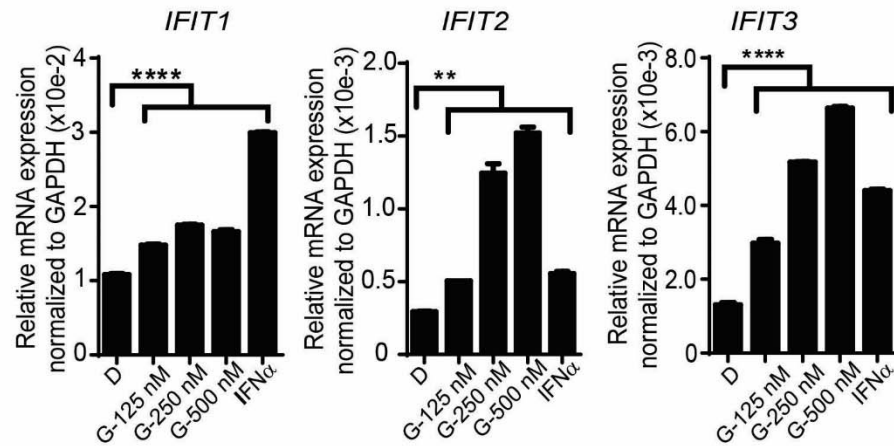
Figure 7. HSP90 inhibition enhances T cell mediated killing of melanoma cells. (A) Tableau depiction of screen results from patient derived cell line 2549. HSP90 inhibitors 1 - 17-DMAG, 2 – BIIB021 and 3 -17-AAG are highlighted. (B) Cleaved caspase 3 percentage following treatment with varying concentrations of ganetespib and autologous TILs in human melanoma cell lines 2549 and 2338. (C) Isobolograms depicting the synergism between ganetespib and T cell killing. Points > 1 indicate antagonism, points = 1 indicate additivity and points < 1 indicate synergism.

P < 0.01; *P < 0.001; ****P < 0.0001. Data are a representation of at least two independent studies.

A



B



C

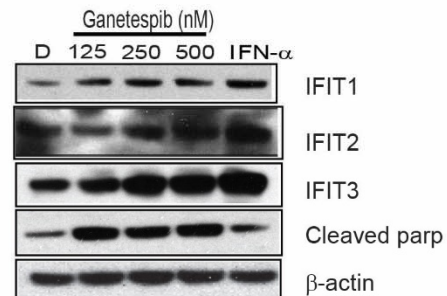


Figure 8. The IFIT genes are essential for the enhanced T cell killing of tumor cells following HSP90 inhibition.

(A) Gene expression analysis of cell lines treated with ganetespib. (B)&(C) Quantitative real time PCR (qRT-PCR) and western blot analysis showing upregulation of IFIT1, IFIT2 and IFIT3 following ganetespib treatment in human melanoma cell line 2400.

P < 0.01; *P < 0.001; ****P < 0.0001. Data are a representation of at least two independent studies.

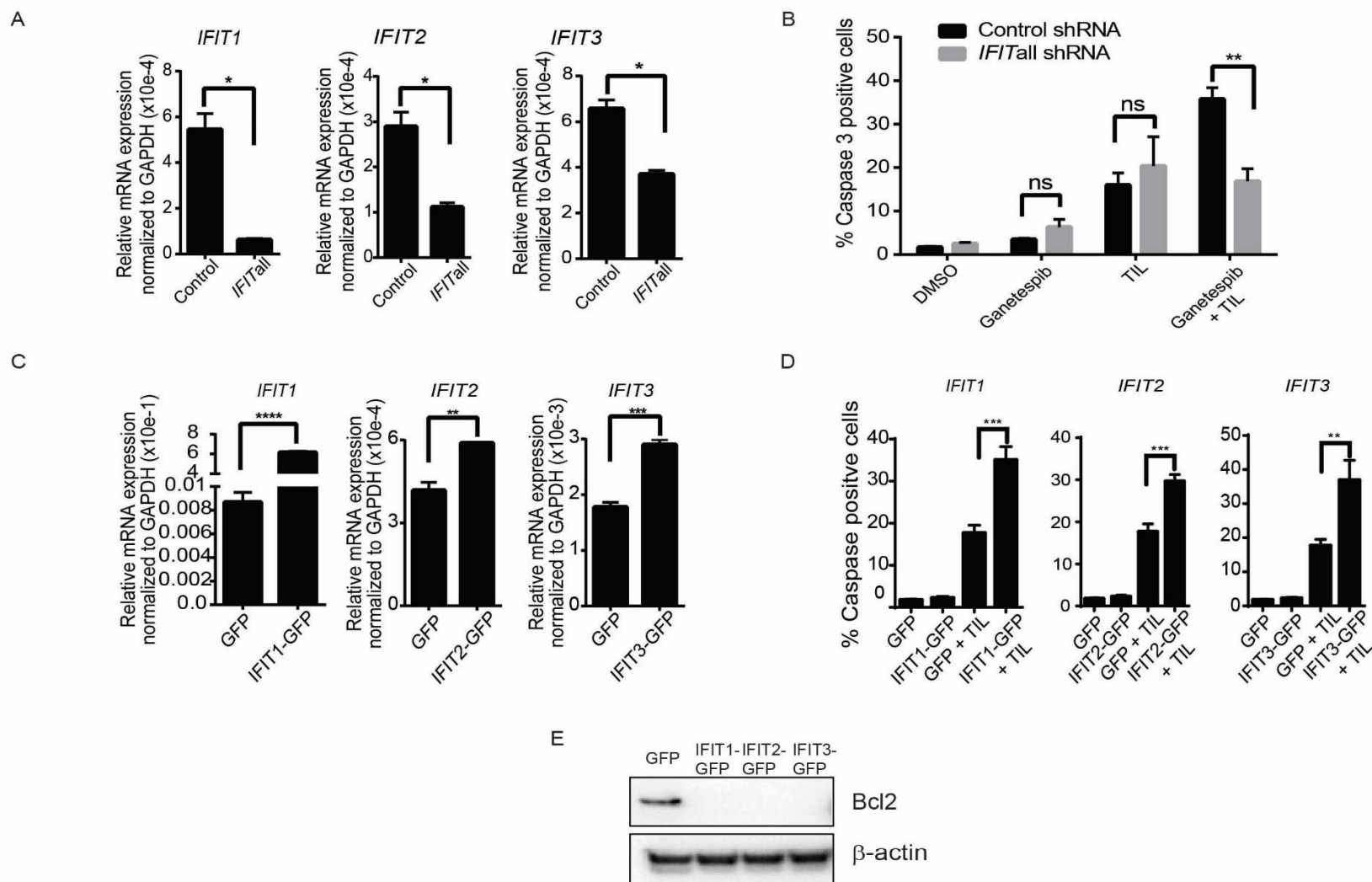


Figure 9. The IFIT genes are essential for the enhanced T cell killing of tumor cells following HSP90 inhibition. A) qRT-PCR to verify silencing of IFIT1, IFIT2 and IFIT3 in 2400. Control = cell line transduced with scrambled shRNA and *IFITall* = cell line transduced simultaneously with IFIT1, IFIT2 and IFIT3 shRNAs. (B) 2400 Control and *IFITall* cell lines treated with ganetespib at 250nM, co-cultured with autologous T cells and assayed for cleaved caspase 3 by flow cytometry. (C) qRT-PCR verifying overexpression of IFIT1, IFIT2 and IFIT3 over GFP control in 2400. (D) 2400 GFP and IFIT overexpressing cell lines co-cultured with autologous T cells and assayed for cleaved caspase 3. (E) Western blots showing a decrease in BCL2 protein after overexpression of IFIT1, IFIT2 and IFIT3. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Data are a representation of at least two independent studies.

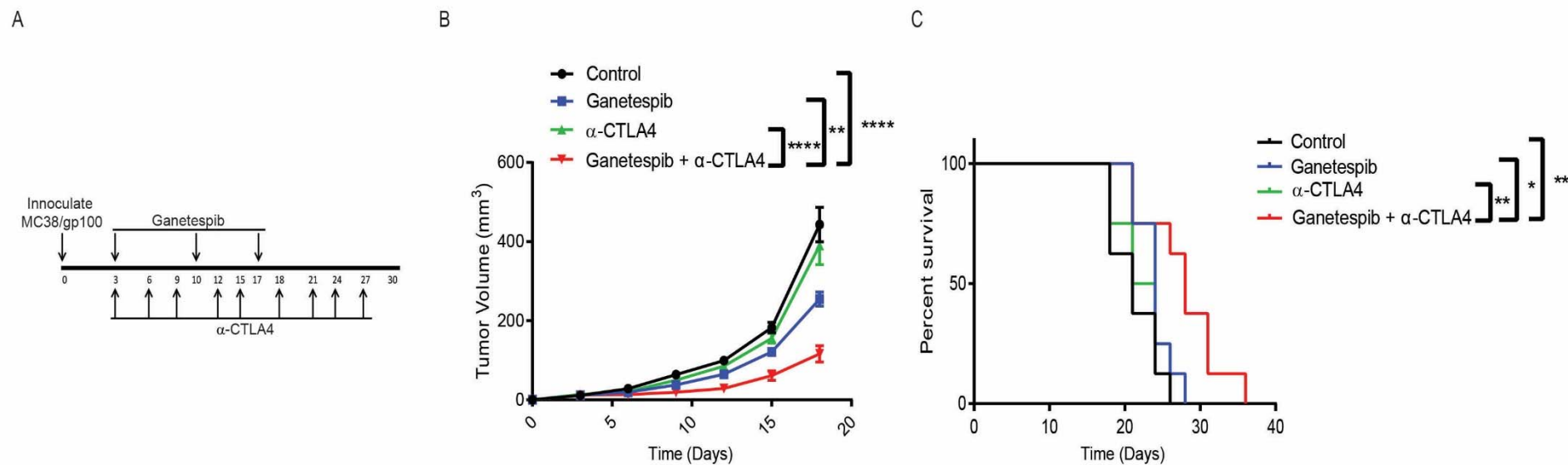


Figure 10. HSP90 inhibition potentiates responses to anti-CTLA4 in vivo.

(A) Treatment schedule: Treatment with ganetespiib and anti-CTLA4 begun at the same time. Ganetespiib was administered at 100mg/kg per mouse once a week and anti-CTLA4 at 100ug per mouse every 3 days. n = 8 mice (B) Survival of animals depicted by Kaplan-Meier curves. n = 8 mice. Vehicle = Solvent + Isotype antibody control. Mice were sacrificed when moribund or when tumor volume reached 1000mm³ or tumors developed ulceration >5mm in diameter. *P < 0.05; **P < 0.01; ***P < 0.001. Data are a representation of at least two independent studies.

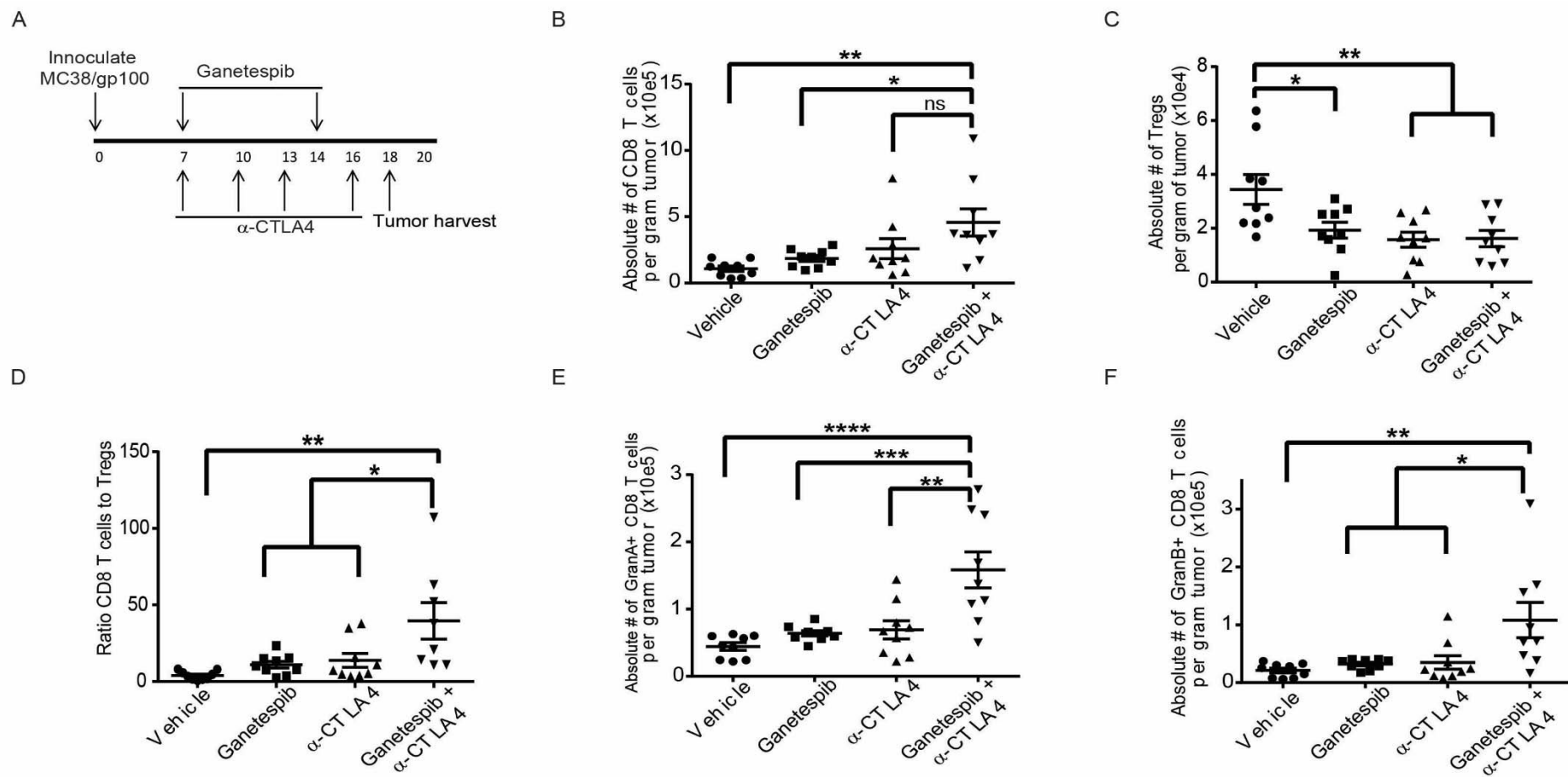


Figure 11: The combination of HSP90 inhibition and anti-CTLA4 treatment enhances the effector function of CD8 T cells

(A) Treatment schedule: ganetespiib was administered at 100mg/kg per mouse once a week and anti-CTLA4 at 100ug per mouse every 3 days. Two doses of ganetespiib and 4 doses of anti-CTLA4 were administered. At day 18, mice were sacrificed, spleens harvested and processed, tumors were harvested and processed for RNA and immune cell infiltration profiled via flow cytometry. (B) Absolute number of CD8 T cells infiltrating the tumor normalized to tumor weight. (C) Absolute number of T regulatory cells infiltrating tumor normalized to tumor weight. (D) Ratio of CD8 T cells to Tregs infiltrating the tumor. (E) Absolute number of granzyme A positive CD8 T cells normalized to tumor weight. (F) Absolute number of granzyme B positive CD8 T cells normalized to tumor weight. n = 9 mice.

*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are a representation of at least two independent studies.

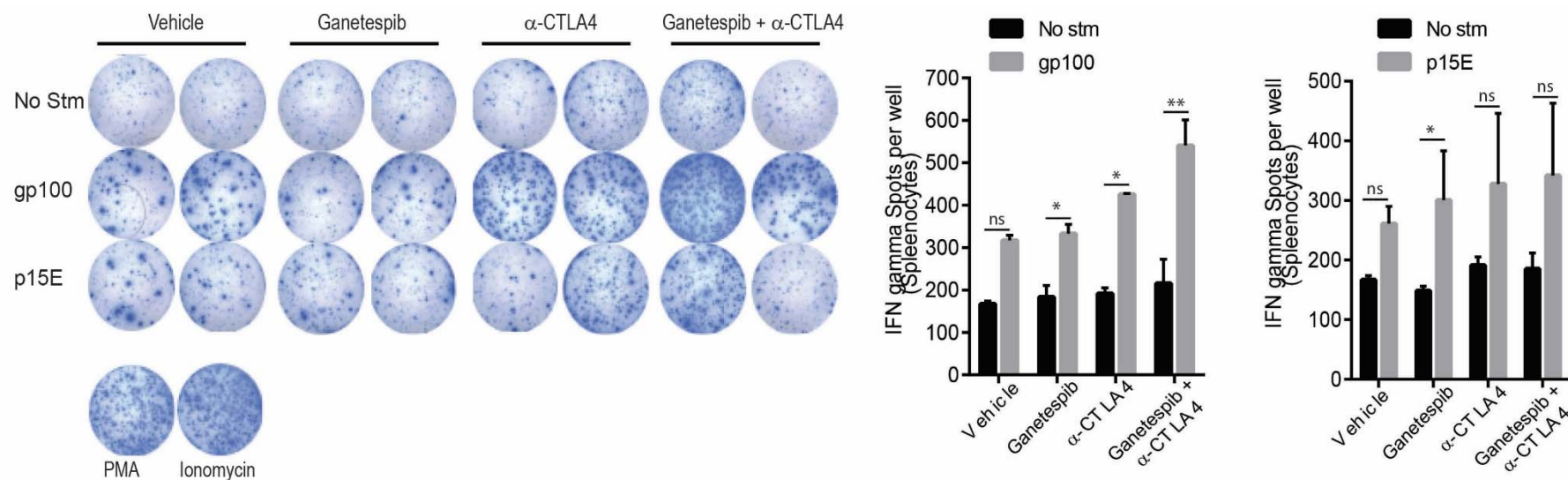


Figure 12: The combination of HSP90 inhibition and anti-CTLA4 treatment enhances the effector function of CD8 T cells

Treatment schedule: ganetespiib was administered at 100mg/kg per mouse once a week and anti-CTLA4 at 100ug per mouse every 3 days. Two doses of ganetespiib and 4 doses of anti-CTLA4 were administered. At day 18, mice were sacrificed, spleens harvested and processed, tumors were harvested and processed for RNA and immune cell infiltration profiled via flow cytometry. ELISPOTS showing gp100 and P15E specific cells from splenocytes of treated mice.

*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are a representation of at least two independent studies.

Chapter 3

Deciphering the role of the transcription factor Runx2 in CD8 T cell function

Abstract

T cells play an important role in cancer immunosurveillance and tumour destruction. By transferring *ex vivo* expanded tumour-infiltrating T cells (TILs), around 50% of melanoma patients can achieve an objective clinical response. While adoptive T-cell therapy (ACT) is very effective in some patients, many patients fail to respond or first respond but then progress. This underscores the need for overcoming the limiting factors in adoptive T cell therapy. One critical limiting factor in ACT is the immunosuppressive tumour microenvironment, which impairs the effector functions of transferred TILs at tumour sites. However, the fact that the underlying suppressive pathways in tumour-reactive T cells have not entirely been identified and characterized has seriously impeded our progress in developing new strategies to help more melanoma patients to achieve favorable clinical outcomes.

Using a murine ACT model transferred CD8 T cells were recovered from the spleen and tumour on day 6 and 13 following adoptive transfer. Gene expression profiling of T cells isolated from the spleen compared those isolated at the tumour site revealed the upregulation of the transcription factor Runx2 in the T cells from the tumour site. Therefore, we hypothesize that the transcription factor Runx2 plays a critical role in regulating the effector functions of tumour-reactive T cells. Runx2 overexpressing Pmel conferred a worse antitumour effect compared to the control cells. Furthermore, the T cells in circulation and at the tumour site were decreased in the mice that received Runx2 overexpressing Pmel compared to the control mice. Further investigation revealed that Runx2 regulated processes in T cells such as cytokine production and differentiation status, processes which are important in controlling tumour growth.

The persistence of T cells in ACT

As mentioned in chapter 1, the persistence and effector function of adoptively transferred TILs is crucial to improving ACT. Durable clinical responses strongly depend on the persistence of the infused TIL. As such, one of the major challenges in ACT is to obtaining ample numbers of tumour-specific T cells with the aptitude to persist long term once infused into patients(120). Reports from several groups indicate that two T cell subsets comprise and contribute the most to long term persistence of TIL. These include the least effector-differentiated memory T cells known as central memory T cells (T_{cm}) and T memory stem cells (T_{scm})(236). Residing in lymphoid organs, T_{cm} and T_{scm} possess robust expansion potential upon antigenic re-encounter as opposed to more differentiated memory T cells. Compared to T_{scm}/T_{cm} effector, effector memory T cells (T_{eff}/T_{em}) respond to antigen with immediate effector function upon homing to tissues though their capacity to regenerate is reduced(237). Furthermore, human T_{em} are divided into CD45RA⁻ or CD45RA⁺ cells. The CD45RA⁺ cells, dubbed Temra, are believed to be the most differentiated memory cells, characterized by decreased proliferative capacity, robust cytotoxic potential, and a higher predisposition to apoptosis(238). T_{scm} have the ability to differentiate into T_{cm} and T_{em}. They show a greater self-renewing potential as demonstrated by a positive correlation of the amount of infused T_{scm} with early expansion after transfer and absolute numbers of long-term persisting cells(239-241). Still, with very low numbers of T_{scm} found in the periphery, extensive expansion would be essential resulting in loss of memory potential. This drawback of low natural frequencies can be circumvented by targeting the Wnt/ β -catenin pathway in naive cells(242, 243). Targeting this pathway results in the arrest of T_{eff} differentiation and the promotion of memory-like CD8 T cells with T_{scm} features. While targeting Wnt signaling seems to be effective in promoting stemness and inhibiting differentiation, it may limit the proliferation and function of CD8 T cells; therefore, further studies are required to evaluate suitability for ACT improvement. Additional methods employed to generate sufficient T_{scm} include cytokine

conditioning (IL-7, IL-15, and IL-21), addition of co-stimulatory antibodies such as 4-1BB and inhibition of Akt signaling during ex vivo expansion of TILs(244, 245).

Transcriptional regulation of T cell differentiation

Over the years, there has been identification of several transcription factors that regulate effector and memory CD8 T cell development. Remarkably, several of these transcription factors function in pairs and form counter-regulatory axes to concurrently produce effector T cells that provide both short- and long-term protection thereby regulating effector and memory cell potential. The two T-box transcription factors T-bet and eomesodermin (EOMES), have been shown to have key roles in the development and function of effector and memory CD8 T cells. In early CD8 T cell activation, T-bet and EOMES direct CTL differentiation by induction of IFN- γ , granzyme B, perforin, CXCR3 and CXCR4(246-249). Initially induced by TCR signaling, T-bet expression is subsequently amplified by IL12 mediated signaling and mammalian target of rapamycin (mTOR) activity in effector CD8 T cells(250). EOMES expression seems to be induced consequently to that of T-bet in a RUNX3-dependent manner. T-bet and EOMES both collaborate to sustain memory CD8 T cell homeostasis via IL-2R β (CD122) expression, enabling IL-15-mediated signaling and the homeostatic proliferation of memory cells. The transcriptional repressor, BLIMP1 (PDRM1) is expressed by effector CD8 T cells and is primarily induced by IL2, IL12 and IL21. BLIMP1 expression declines as memory CD8 T cells mature ensuing pathogen clearance. As such BLIMP1 is expressed at the lowest levels in Tcm cells. BLIMP1 is part of a transcriptional program promotes terminal effector cells and enhances CTL functions. A well characterized antagonist of BLIMP1 activity, is the transcription factor BCL6. The expression of BCL6 has been shown to be inversely correlated with that of BLIMP1 in both effector and memory CD8 T cells. CD8 T cells that Overexpression of BCL6 in CD8 T cells results in the generation of increased numbers of Tcm cells(251). Another pair of transcription factors with important roles in effector and memory Cd8 T cell generation are inhibitor of DNA binding 2 (ID2) and ID3. ID2 and ID3

seem to perform their roles in effector CD8 T cells in a separate but temporal manner. While ID2 promotes the survival of effector CD8 T cells in the cell transition from naïve to effector, ID3 promotes their survival from effector to memory cell transition(248). The transcription factors STAT3 and STAT4 are also important in effector and memory CD8 T cells generation. All-in-all CD8 T cell effector and memory differentiation is a process highly influenced by a delicate balance of the expression and/or repression of several transcription factors (Figure 13). Effector CD8 T cells acquire a more terminally differentiated phenotype associated with decrease in proliferative capacity and survival as the expression or activity of T-bet, BLIMP1, ID2 and STAT4 increases. In contrast, EOMES, BCL-6, ID3, TCF1 and STAT3, prevent terminal differentiation and/or help to maintain memory cell properties, such as long-term survival, proliferative potential, developmental plasticity and the ability to self-renew.

Runx-related transcription factor 2

The Runx-related transcription factor (Runx) family consists of three members, Runx1, Runx2 and Runx3, characterized by a conserved DNA binding Runx homology domain (RHD). The RHD mediates formation of a heterodimer with the common subunit CBF β , which itself does not interact with DNA but increases the transcriptional activity of the Runx proteins(252). Runx1 and Runx3 are important in T cell development, particularly in commitment of double positive T cells to the CD8 T cell lineage by activation of CD8 and epigenetic silencing of CD4 in double negative T cells respectively, thereby favoring the development of CD8 T cells (253-255). Runx3 is also important in the development of effector and memory cells as it induces T-bet expression. Runx2 on the other hand is well known for its indispensable role in bone development, primarily as a regulator of osteoblast differentiation. Early in bone development, activation of Runx2 commits pluripotent mesenchymal stem cells to the osteoblast lineage and activates downstream genes which are important in osteoblast differentiation. In addition, Runx2 controls mature osteoblast differentiation through inducing an anti-proliferative state thereby retaining cells in the G1 phase

of the cell cycle(256). In addition, Runx2 is also essential for chondrocyte maturation(257). As such, heterozygous loss of function of Runx2 through mechanisms such as missense mutations, deletions, point mutations and insertion results in a rare genetic condition in humans known as cleidocranial dysplasia (CCD) characterized by lack of intramembranous bone formation, hypoplasia or aplasia of the clavicle(258, 259). Mice heterozygous for Runx2 display lack clavicular development and mirror the symptoms seen their human counterparts (258). Although Runx2 has been extensively studied in bone development, little is known about its role in the hematopoietic system. Recent studies indicate the importance of Runx2 in the development and migration of plasmacytoid dendritic cells from the bone marrow to the periphery in a CCR5 dependent manner(260). Runx2 has been shown to be expressed in developing and mature T cells, however, its role in the normal CD8 T cells has not yet been elucidated (261). In this chapter, I evaluated the transcription factor Runx2 for its ability to regulate effector/memory CD8 T cells differentiation.

Results

Runx2 is upregulated in Pmel T cells at the tumour site

To mimic the clinical setting of adoptive T cell therapy and characterize the phenotype of tumour-reactive T cells at the tumour, we used a murine ACT model in which B16, a gp100-expressing tumour cell line was implanted in mice and treated with transgenic Pmel T cells which recognize gp100 in the context of H-2D^b (262, 263) (Figure 14A&B). On day 6 and 13 following T cell transfer, transferred Pmel T cells were recovered from the spleen and tumour. To interrogate novel pathways which may inhibit the functions of tumour-reactive T cells at the tumour site, microarray and genome-wide gene expression analyses, were used to characterize the differential expression profiles among Pmel T cells from different groups. Results demonstrated that the profile of Pmel T cells isolated from the spleen of the tumour bearing mice differed significantly from the profile of T cells isolated from the tumour, but was similar to the profile of T cells isolated from the spleen of the tumour free mice (Figure 14C). Gene Set Enrichment Analysis(GSEA)(264) demonstrated that the phenotype of tumour-reactive T cells is positively correlated to the profile of exhausted T cell defined in previous study(265)(Figure 14D), indicating the presence of suppressive pathways in tumour-reactive T cells at tumour sites. To select candidate pathways for further study, we applied the guidelines listed in Figure 14E and found that 720 genes were differentially expressed by T cells recovered from the tumour site, when compared with those recovered from the spleen. One of the gene sets found to be differentially expressed by ingenuity pathway analysis were genes involved in osteoblast differentiation (Figure 14F) including the transcription factors (TFs) Tcf7 and Runx2 (a gene whose role has not yet been described in T cells). This suggested that Runx2 may play a role in regulating T cell function at the tumour site.

Runx2 expression impairs Pmel T cell antitumour response

The transcription factor Runx2 is well known for its role in osteoblast differentiation, however the role of Runx2 has not been previously described in T cells. Given that transcription factors, including Runx1 and Runx3, have been shown to play a critical role in the differentiation and effector function of CD8 T cells, we hypothesized that Runx2 may regulate several processes in T cells including effector function, survival, proliferation and as a result the antitumour effect of CD8 T cells(266, 267). To investigate the role of Runx2 in the antitumour effect of CD8 T cells, Runx2 was overexpressed using a retroviral vector system. We were unable to silence Runx2 using small hair pin RNAs (shRNAs) and so we used Runx2 heterozygous and homozygous knockout cells generated from fetal liver transplants as described in Supplemental figure 7. Runx2 overexpression and deficiency was confirmed by qrtPCR and flow cytometry (Supplemental figure 8A&B). Runx2 overexpressing and deficient cells were adoptively transferred into B16 tumour bearing mice and their antitumour activity was assessed by monitoring the tumour size. Mice that received the Runx2 overexpressing Pmel had larger tumours compared to the control cells and mice that received the Runx2 deficient Pmel had smaller tumours compared to the mice that received WT Pmel (Figure 15A). Furthermore, there was no difference in the number of circulating Pmel or number of Infiltrating Pmel in the mice that received Runx2 deficient Pmel compared to wild type cells (data not shown). But, the T cells in circulation and at the tumour site were decreased in the mice that received Runx2 overexpressing Pmel compared to the control mice, suggesting that Runx2 negatively regulates the ability of CD8 T cells to control tumour growth (Figure 145&C).

The transcription factor Runx2 regulates effector differentiation of P mel T cells

The differentiation status of T cells (effector vs memory) plays an important role in their antitumour effect(268, 269). To investigate whether Runx2 expression affect the differentiation status of CD8

T cells, control, Runx2 over, WT and Runx2 deficient Pmel T cells were cultured in IL2 supplemented medium for 24hrs without any stimulation (Supplemental Figure 9). Gene expression analysis of the cultured cells revealed differential expression of genes pathways involved in T cell effector vs memory differentiation. Amongst these genes were the following classes of genes(270) : Effector/differentiation – Eomes, granzymes, perforin, Trafficking – CXCR3, CXCL10 and Differentiation – CD62L (SELL). In addition genes such as Spp1 and Tgfb β 2 known to be regulated by Runx2 were also differentially expressed (Figure 16A). The expression of multiple markers was confirmed by flow cytometry with markers such as CD62L, Tcf7, Cxcr3 expression being decreased in the Runx2 overexpressing cells and increased in Runx2 deficient cells. While, Lag3 and Blimp1 (Prdm1) expression were increased in Runx2 overexpressing cells and decreased in Runx2 deficient cells, consistent with reported markers that differentiate effector cells from memory cells (Figure 16B&C). These data suggest that Runx2 expression promotes effector (possibly terminal effector) cell differentiation. Taken together, Runx2 overexpression promotes more of a terminal effector phenotype while Runx2 deficiency promotes more of a memory phenotype which is beneficial for antitumour immunity.

Runx2 expression promotes effector functions reminiscent of terminal effector CD8 T cells

As mentioned above, Runx2 overexpression promotes more of an effector phenotype. As such effector functions such as cytokine production and cytotoxic ability should be affected by Runx2 perturbation. Terminal effector cells are characterized by a decrease in their ability to produce effector cytokines, decreased proliferation, increased apoptosis and increased cytotoxicity. To test whether Runx2 perturbation affected cytokine production, control, Runx2 overexpressing, WT and Runx2 deficient Pmel cells were stimulated in the presence of gp100 pulsed DCs, B16 and mc38gp100 tumours *in vitro* for 24hrs. Cytokine secretion was assessed via Luminex and flow cytometry. Overexpression of Runx2 lead to a decrease in the secretion of the effector cytokines IL2, TNF- α , GM-CSF and IFN- γ while a deficiency of Runx2 had the opposite effect.

Conversely, Runx2 overexpression lead to an increase and/or sustained production of the suppressive cytokines IL10 and Spp1 while a Runx2 deficiency had the opposite effect, suggesting that Runx2 expression suppressed effector cytokine production (Figure 17A and Supplemental figure 10A&B). To evaluate whether, the decrease in cytokine production was as a result of decreased cell survival, control, Runx2, WT and Runx2 deficient cells were cultured for 24hrs and stained for annexin V. Although Runx2 deficiency had no effect on the survival of the Pmel T cells, Runx2 overexpression lead to increased annexin V expression, suggesting that Runx2 expression could promote apoptosis (Figure 17B). The proliferation of these cells was also evaluated via Brdu incorporation and a similar result was obtained. Runx2 deficiency did not affect Pmel proliferation, however, Runx2 overexpression lead to a decrease in Pmel proliferation (Figure 17C). Furthermore, because many of the granzyme genes were differentially expressed in the gene expression analysis, the cytotoxic ability T cells was evaluated via T cell mediated lysis of tumour cells. Runx2 overexpressing pmel induced higher levels of caspase 3 in tumour cells. Meanwhile, Runx2 deficient Pmel mediated decreased lysis of tumour cells (Figure 17D). Taken together, the effector phenotype displayed by Runx2 overexpressing Pmel compared to Runx2 deficient Pmel suggest that Runx2 promotes terminal effector differentiation of CD8 T cells while Runx2 deficiency promotes a memory phenotype.

Discussion and future directions

In this study, we identified the transcription factor Runx2 as a gene upregulated in CD8 T cells at the tumour site compared to the spleen. Runx2, whose role has been well established in osteoblast biology, has not yet been elucidated in T cells. Studies over the years have shown that subsets of effector and memory CD8 T cells can be differentiated by the cell surface and intracellular markers they express, their trafficking patterns, as well as their ability to perform effector functions. Terminally differentiated CD8 T cells or terminal effector CD8 T cells (TEF) express low levels of the cell surface markers CD62L and Cxcr3 amongst others, while expressing high levels of inhibitory receptors such as Lag3 and Klrp1. They also display low levels of the transcription factor Tcf7 and high levels of the transcription factor Blimp1. They have decreased effector cytokine secretion, increased inhibitory cytokine secretion and increased cytotoxicity. Central memory T cells (Tcm) on the other hand display an opposite phenotype from TEF. Perturbation of Runx2 in CD8 T cells revealed that Runx2 expression promotes the differentiation of a TEF phenotype, characterized by decreased effector cytokine production, decrease proliferation, decreased survival and increased cytotoxicity, as well as surface and intracellular markers consistent with those expressed by TEF CD8 T cells (Table 1). Meanwhile, Runx2 deficient T cells display more of a Tcm phenotype characterized by increased effector cytokine secretion and decrease cytotoxic, as well as expression of surface and intracellular markers consistent with those expressed by Tcm CD8 T cells.

Although Runx2 overexpressing Pmel T cells kill better, their antitumour activity was impaired in our *in vivo* ACT studies. This seems counter intuitive, as cells with better cytotoxicity should control tumours better. This could be explained by the fact that Runx2 overexpressing T cells have decreased levels of the chemokine receptor Cxcr3, important for trafficking into tumours. The chemokines Cxcl9 and Cxcl10 are the ligands for Cxcr3 and are usually expressed in the tumour microenvironment in an ongoing antitumour immune response. Cxcl9 and Cxcl10

are produced by myeloid cells in the tumour microenvironment as well as tumour cells themselves. In addition, once T cells traffick into the tumour and interact with tumour cells, they release IFN- γ which further upregulate these chemokines and induce a feedback loop chemokine and cytokine expression bringing in more T cells. As observed in our *in vitro* studies, IFN- γ secretion was also decreased in the Runx2 overexpressing cells. The decreased expression of Cxcr3, coupled with decreased effector cytokine production could contribute greatly to the decreased T cell infiltration into the tumour and hence impaired antitumour effect. The Runx2 deficient T cells on the other hand express higher levels of Cxcr3 and produce higher levels of effector cytokines compared to the WT cells. Although they have decreased ability to kill, this may be compensated for by the increased effector cytokine secretion, which can also have direct inhibitory effects on the tumours cells as well as recruiting other immune cells. In contrast, Runx2 overexpression leads to the production of inhibitory cytokines such as IL10 and Spp1 (a Runx2 regulated cytokine). IL10 has been reported to be part of a fully/terminally differentiated characteristic of CD8 T cells. In other words, TEF CD8 T cells produce IL10 as a regulatory mechanism to suppress CD8 T cell immune response in an autocrine manner. In addition IL10 has also been shown to be produced by highly dysfunctional CD8 T cells. Spp1 on the other hand has been shown to promote the differentiation of terminal effector CD8 T cells, although the source of Spp1 in this study was from DCs. Here we show that CD8 T cells themselves produce copious amounts of Spp1 following Runx2 overexpression. Runx2 deficient cells on the other hand express very low levels of IL10 and Spp1. It is possible that these cytokines also contribute to the potentiation of the TEF phenotype displayed by Runx2 overexpressing CD8 T cells. A noteworthy point is the fact that Tcm regain their ability to lyse tumour cells rapidly once they re-encounter antigen. The observation that Runx2 deficient T cells had decreased cytotoxicity suggests that Runx2 may directly regulate T cell cytotoxicity and is required in moderate amounts once Tcm re-encounter antigen.

Under various stimulatory conditions, Runx2 upregulation was observed, suggesting that Runx2 expression may be normally induced down stream of TCR signaling (Supplemental Figure 11). However, drawing parallels from osteoblast biology, some of the factors known to induce and potentiate Runx2 expression during osteoblast differentiation or promote osteoblast differentiation are suppressive to CD8 T cells. Factors such as Spp1, Tgf- β and IL10 are examples of such factors. The tumour microenvironment is known to be immunosuppressive with tumour cells, as well as other suppressive immune cells making these factors. It is possible that induction of Runx2 downstream of TCR signaling may be a naturally occurring phenomenon and necessary for the acquisition of cytotoxic functions. However, the expression of inhibitory factors such as Tgf- β , may further potentiate Runx2 expression promoting the differentiation of CD8 T cells to TEF cells. Notably, Runx2 upregulation can also lead to the secretion of these suppressive factors by CD8 T cells themselves, potentiating this suppressive environment. Overall these correlations between our *in vitro* and *in vivo* studies provide some hypotheses as to why the antitumour effect of the Runx2 overexpressing Pmel cells was impaired. However, these observations will have to be confirmed by *in vivo* studies evaluating the survival, trafficking and cytokine secretion. In addition, *in vivo* studies will be needed to confirm the potentiation of the suppressive effects by factors such as Tgf- β . Finally, combination studies of ACT with other costimulatory antibodies, in the context of Runx2 overexpression may be necessary to find situations where the cytotoxic promoting ability Runx2 can be harnessed while limiting its suppressive effects.

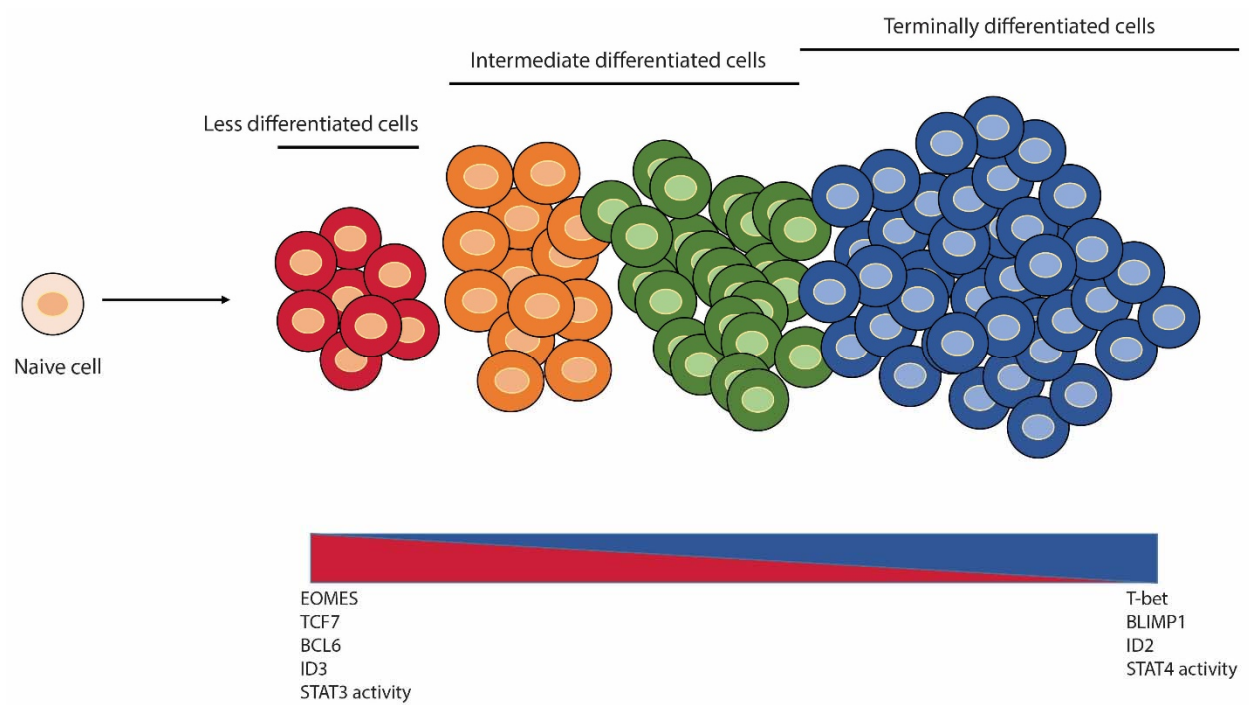


Figure 13: Transcription factors expressed at various stages of T cell differentiation

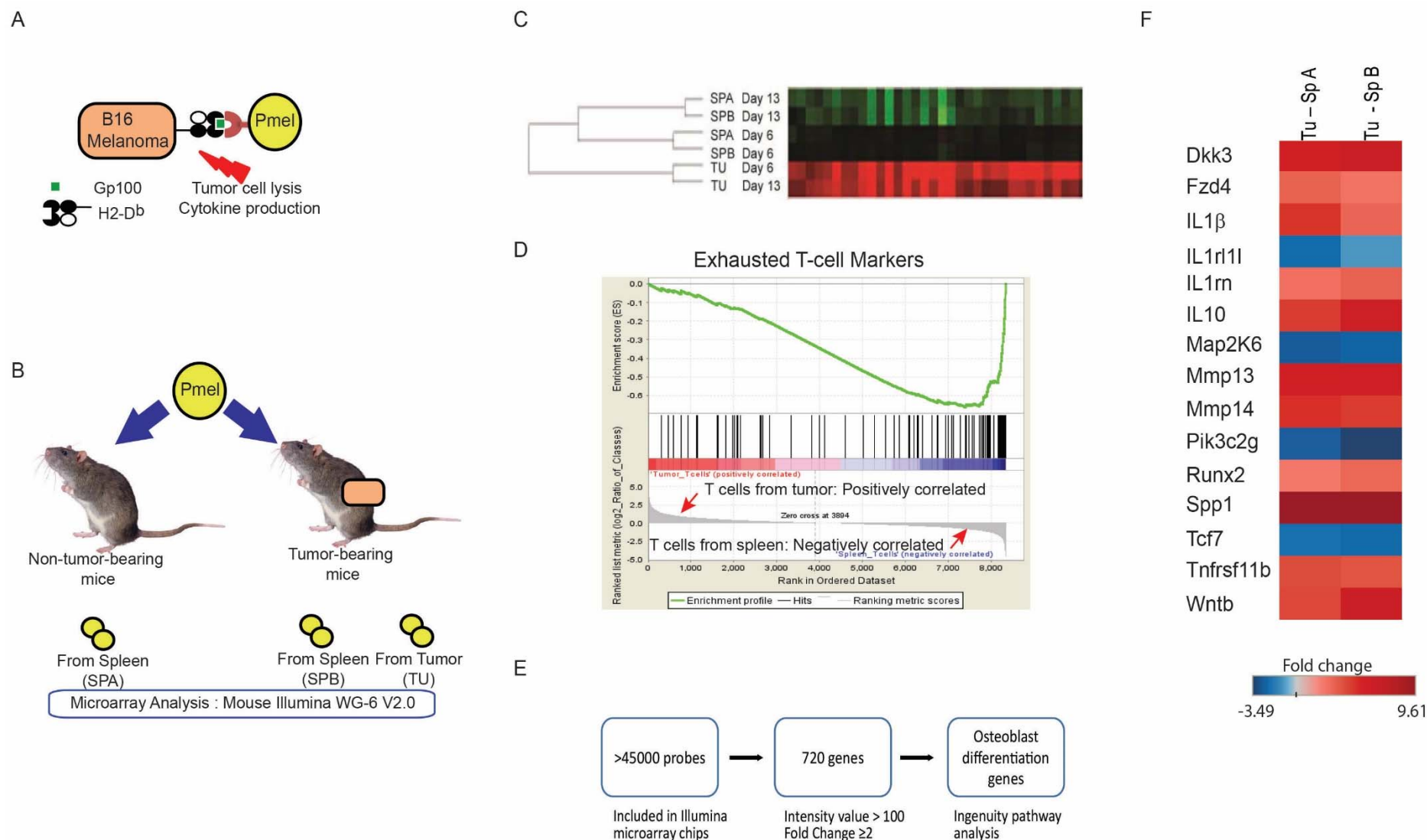


Figure 14: Runx2 is upregulated in Pmel T cells at the tumour site. A) ACT model utilized. B) Experimental design for Pmel cells collected for microarray analysis. Transferred Pmel re-isolated from spleens and tumours on Day 6 and Day 13 following ACT. C) Gene expression pattern (GEP) of T cells from spleen of tumour bearing and tumour free mice similar but different from tumour. D) GSEA showing correlation of gene expression pattern of Pmel from the tumour and spleen with exhaustion markers. E) Guidelines for candidate gene selection. F) Osteoblast differentiation gene set enriched in Pmel at tumour site. Tu-SpA = GEP from tumour normalized to spleen from tumour free mice. Tu-SpB = GEP from tumour normalized to spleen from tumour bearing mice

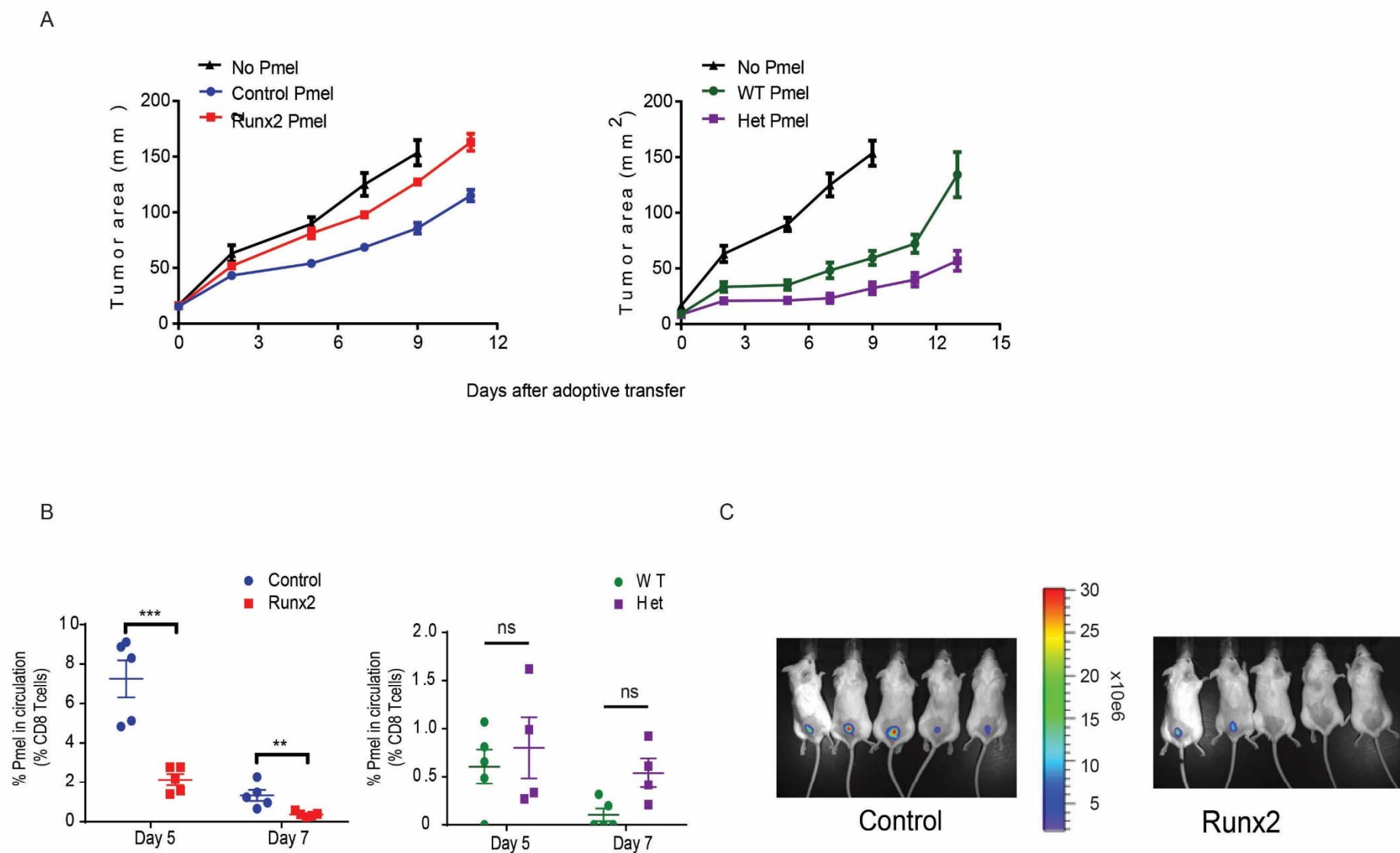


Figure 15: Runx2 expression impairs CD8 T cells antitumour response. A) B16 bearing mice treated with adoptive transfer of Runx2 perturbed Pmel cells. Tumour volumes over time. B) Pmel in circulation measured as percentage of total CD8 T cells. C) Pmel T cells co-transduced with luciferase firefly plasmid to track them *in vivo* Control = GFP control, Runx2 = Runx2 overexpression, WT = Untransduced control, Het = Runx2 heterozygous.

P < 0.01; *P < 0.001. Data are a representation of at least two independent studies.

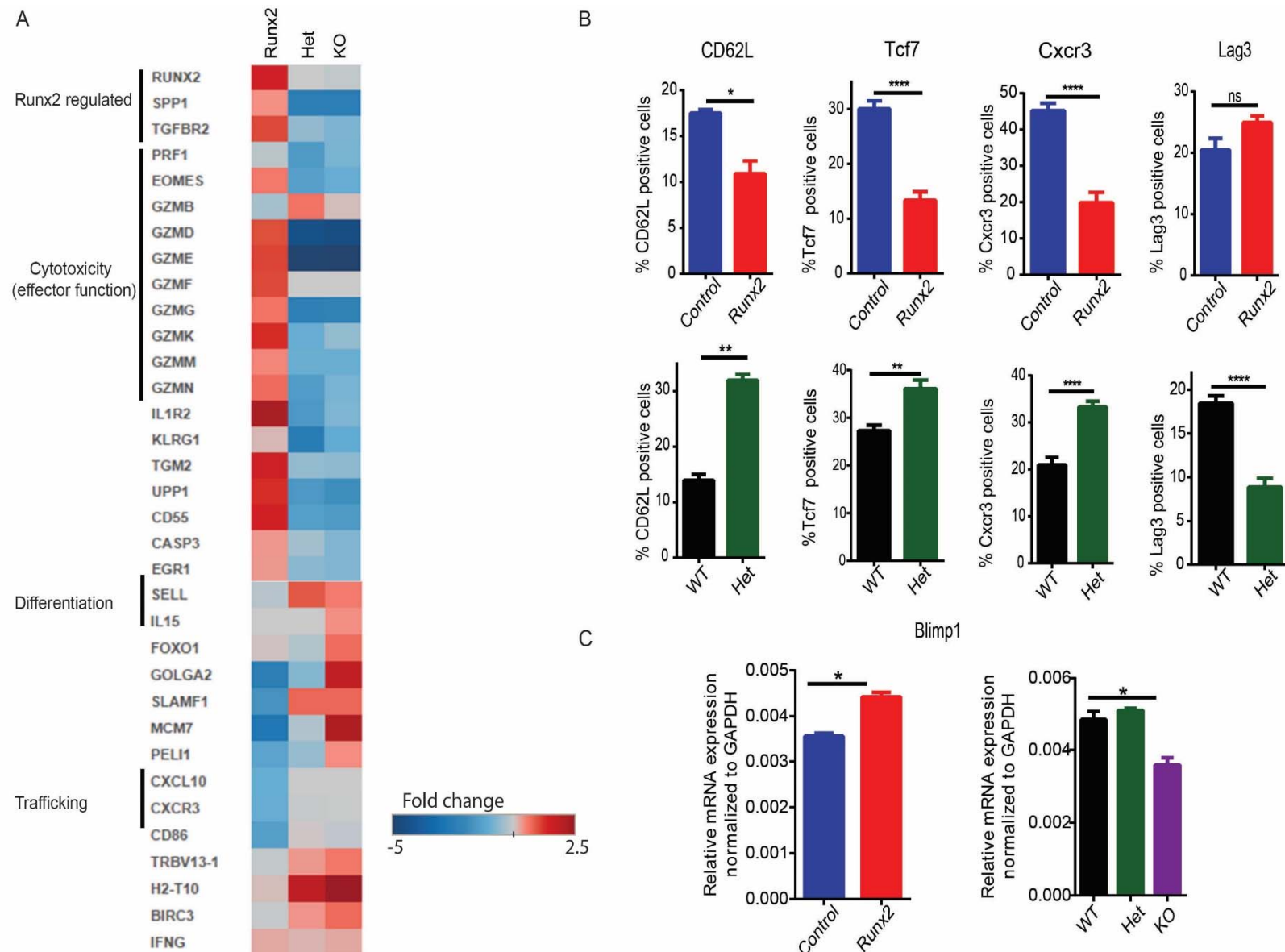


Figure 16: The transcription factor Runx2 regulates effector differentiation of CD8 T cells. A) Gene expression analysis of Runx2 perturbed Pmel cells cultured for 24hrs without stimulation. Runx2 = Runx2 overexpressing cells normalized to GFP control expressing, Het = Runx2 heterozygous cells normalized to untransduced WT cells, KO = Runx2 homozygous deleted cells normalized to untransduced WT cells. B) Expression of different cell surface makers in Runx2 perturbed Pmel cells. C) Blimp1 expression by quantitative real time PCR. Control = GFP control, Runx2 = Runx2 overexpression, WT = Untransduced control, Het = Runx2 heterozygous. *P < 0.05, **P < 0.01, ***P < 0.001; ****P < 0.0001.

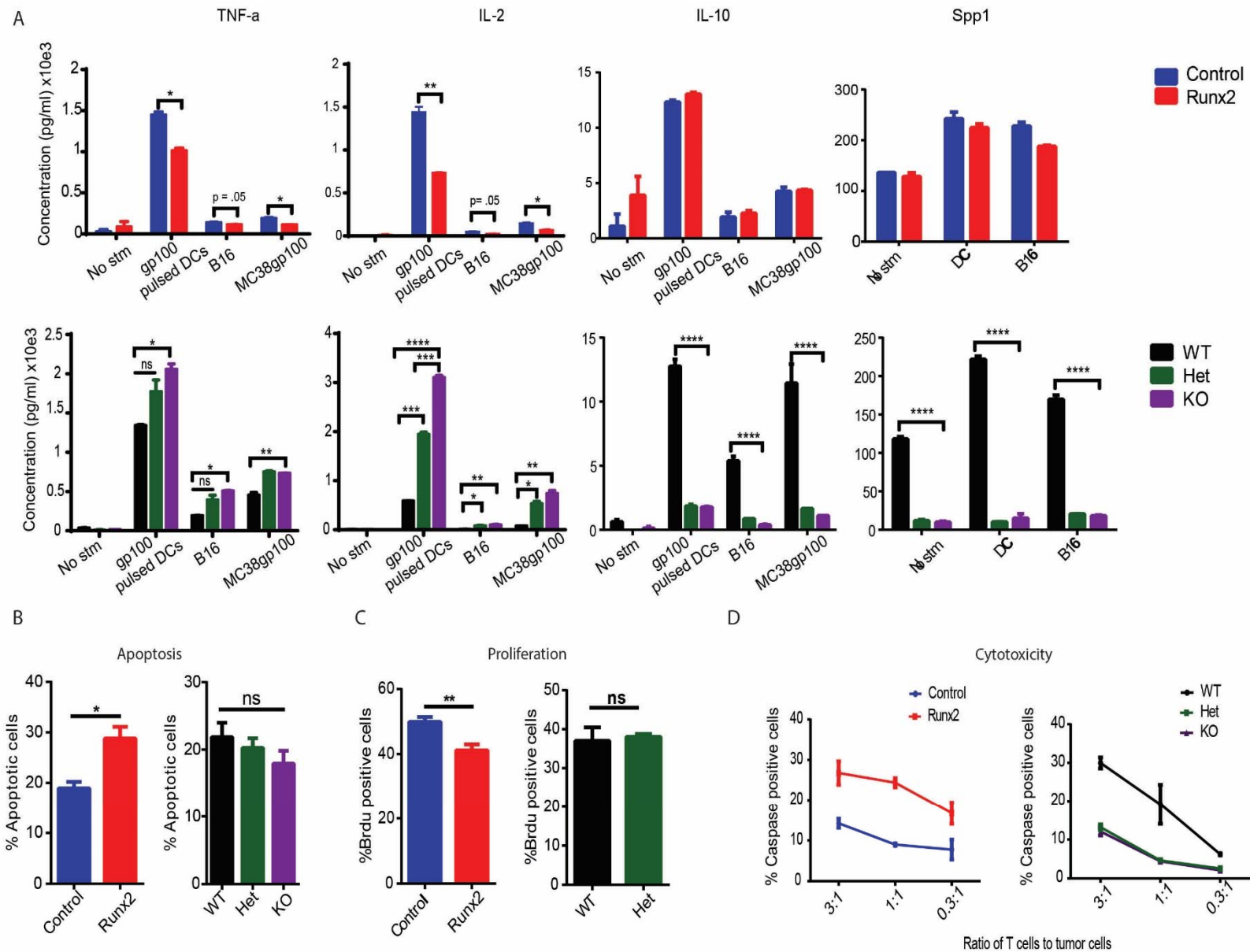


Figure 17: Runx2 expression promotes effector functions reminiscent of terminal effector CD8 T cells. A) Cytokine secretion by Pmel T cells in the presence of no stimulation (No stm), gp100 pulsed DCs B16 and MC38gp100 tumour cells. B) Percentage of apoptotic cells measured by Annexin and 7-AAD staining. C) Pmel T cell proliferation at 24hrs of culture measured by Brdu incorporation. D) Pmel T cell mediated killing of MC38gp100 tumour cells measured by caspase 3 cleavage. Control = GFP control, Runx2 = Runx2 overexpression, WT = Untransduced control, Het = Runx2 heterozygous, KO = Runx2 homozygous. *P < 0.05, **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are a representation of at least two independent studies

	Runx2 overexpression	Runx2 deficient
Surface Phenotype	CD62L ^{lo} CXCR3 ^{lo} LAG3 ^{hi}	CD62L ^{hi} CXCR3 ^{hi} LAG3 ^{lo}
Cytokines	IFN γ ^{+/-} TNF α ^{+/-} IL2 ⁻ Spp1 ⁺⁺ IL10 ⁺⁺	IFN γ ⁺ TNF α ⁺ IL2 ⁺ Spp1 ⁻ IL10 ⁻
Transcription factors	Blimp1 ^{hi} TCF7 ^{lo}	Blimp1 ^{lo} TCF7 ^{hi}
Cytotoxicity	Increased	Decreased
Proliferation	Decreased	No change
Apoptosis	Increased	No change
Phenotype	Terminal effector T cells	Central memory T cells

Table 1: Pmel T cell phenotype in the context of Runx2 perturbation

Chapter 4

Materials and Methods

Study approval.

6-12 week old C57BL/6 mice were purchased from the Charles River Frederick research model facility (Bethesda, MD). Mice were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee. The MC38/gp100 cell line was established as previously described(130). B16 tumour cell line was obtained from ATCC. Human melanoma cell lines 2338, 2400, 2549, 2559 and their autologous T cells were established from metastatic melanoma patients enrolled in the adoptive cell therapy clinical trial at M.D. Anderson Cancer Center according to previously described protocols (271). All cell lines were maintained in complete cell culture medium as previously described (193).

Compound screen

A library of 850 bioactive compounds was purchased from Selleckchem. Patient derived human melanoma cell lines were labelled with DDAO cell tracker dye. The labelled cells were then treated with either 1 uM of each compound or DMSO alone for 24 hrs at 37°C in a 96 well format or treated first with the compound or DMSO, washed off and co-cultured with autologous T cells at a predetermined ratio for 3 hrs. The cells were then assayed for cleaved caspase 3 via flow cytometry as previously described (272). A comboscore was calculated based on observed changes in the percentage of T cell–induced apoptosis in tumour cells with or without compound treatment. Compounds that enhance the sensitivity of tumour cells to T cell–mediated killing have comboscores >1. $\text{Comboscore} = ((\text{Compound\&Tcells} - \text{Compound})/(\text{Tcells} - \text{DMSO}))^2$.

Gene expression profiling (GEP) and analysis

The patient derived human melanoma cell lines 2338, 2400, 2549 and 2559 were treated in duplicate with DMSO as a control or 125 nM of ganetespib for 24hrs at 37°C. Total RNA was collected for GEP on Illumina human HT12v4.0 arrays as previously described (273). For each cell line and gene probe, the log2 values of ganetespib duplicates were averaged and subtracted

by the log2 values of DMSO samples. The average of these subtracted values, across all 4 lines, was then used to create a gene rank for analysis by gene set enrichment analysis (GSEA) software, which uses a Kolgorimov-Smirnov statistic to determine the significance of distribution of a set of genes within a larger, ranked data set(264). GEP data were also analyzed using Ingenuity Pathway Analysis software, which scores canonical pathways by their consistent change across all samples, with a positive z score indicating an increase in expression or activation of the canonical pathway, and a negative z score indicating a decrease.

Quantitative real time PCR

Total RNA was extracted using RNAeasy plus mini kit (QIAGEN). 2ug of RNA was reverse transcribed to cDNA using the High Capacity RNA-to-cDNA kit (Thermofisher). Quantitative real time PCR was performed using the Taqman gene expression assay system according to according to manufacturer's instructions (Life Technologies). A list of the gene expression assays used is provided in the Supplementary Table 1. Samples were normalized to GAPDH expression level using $2^{-\Delta\Delta CT}$ method.

Western blots

Samples were lysed in RIPA lysis buffer system (Santa Cruz) according to manufacturer's instructions. 50ug of protein in cell lysates were separated in 4-20% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with the following primary antibodies against IFIT1 (Cell Signaling), IFIT2 (Abcam), IFIT3 (Abcam), cleaved PARP (Cell signaling), BCL2 (Cell signaling) and β -actin (Cell Signaling). Anti-rabbit and anti-mouse IgG antibodies tagged with horseradish peroxidase (Cell signaling) were used.

Generation of ORF-expressing tumour cells for in vitro T cell-killing assay

Viral particles containing ORF gene-of-interest were used to infect patient-derived melanoma cells. To test the effect of overexpression of *IFIT* genes, freshly transduced cells were co-cultured

with autologous T cells. ORF-positive cells were gated based on the expression of tagged GFP via flow cytometry analysis.

Generation of shRNA-expressing tumour cells lines for in vitro T cell-killing assay

Viral particles containing either control shRNA or shRNAs of gene-of-interest were used to infect patient derived melanoma cells. Stable cell lines expressing shRNAs targeting the *IFIT* genes were generated by 2-week puromycin treatment after viral transduction. These established stable cell lines were used for *in vitro* experiments. To test the effect of the knockdown of the *IFIT* genes to the synergistic effect of ganetespib and T cell killing, freshly transduced cells were treated with either ganetespib or DMSO for 24hrs and assayed as described above.

Treatments, immune cell tumour infiltration analysis and ELIPOT analyses

The HSP90 inhibitor ganetespib was provided by Synta Pharmaceuticals (Lexington, MA). Mice were treated with a dose of 100mg/kg once weekly. Anti-CTLA-4 (9H10) was purchased from BioXcell and was administered every 3 days at a dose of 100ug/mouse. Isotype antibody control used was Polyclonal Armenian Hamster IgG. Treated mice were sacrificed on day 18 following tumour inoculation and tumours and spleens were harvested. Tumours were dissected into fragments by cutting, digested in tumour digestion buffer for 2 hours at 37°C and filtered through 45µm nylon mesh. The tumour digestion buffer was made by dissolving 1mg/ml collagenase, 100µg/ml hyaluronidase and 20mg/ml Dnase (Sigma-Aldrich, St. Louis, MO) in RPMI medium. Cell suspensions were stained for intracellular and extracellular protein markers of interest. Stained samples were acquired on a BD LSRFORTESSA X-20 instrument and data analyzed using Flowjo software. Staining antibodies were as follows: Anti-CD45 (30-F11, Tonbo Biosciences), anti-CD8 (53-6.7, Tonbo Biosciences), anti-CD3 (145-2C11, Tonbo Bioscience), anti-CD4 (RMA-5, Tonbo Biosciences), anti-Foxp3 (FJK-16s, eBioscience), anti-CD25 (PC61.5, eBioscience), anti-Gr1 (RB6-8C5, Tonbo Biosciences), anti-Ly6C (HK1.4, Biolegend), anti-

CD11b (M1/70, Tonbo Biosciences), anti CD11c (N418, Tonbo Biosciences), anti-F4/80 (BM8.1, Tonbo Biosciences), anti-granzyme B (GB11, BD Bioscience), anti-granzyme A (GzA-3G8.5, Affymetrix Inc.), anti-Ki67 (SolA15, Affymetrix Inc.) and anti-cleaved caspase 3 (550821, BD Bioscience). Single cell suspensions of splenocytes were prepared and erythrocytes depleted using ACK lysis buffer (Life technologies). The murine IFN-gamma single-color enzymatic ELISPOT assay kit from Immunospot was used to assess IFN-gamma producing cells. Splenocytes from 3 mice with similar tumour weights were combined within each treatment group and 300,000 splenocytes were incubated with either DMSO, gp100 peptide (KVPRNQDWL) at 1ug/ml or p15E peptide (KSPWFTTL) at 1ug/ml for 24hrs at 37°C and assayed according to the manufacturer's instructions. Spots were counted and quantified using the CTL Immunospot reader.

Fetal liver transplant

Fetal liver transplant was performed as previously described. Pmel mice were crossed with Runx2^{+/-} mice to obtain Runx2^{+/-} Pmel. Runx2 homozygous mice (Runx2^{-/-}) mice die at birth due to an arrest in osteoblast maturation(259, 274, 275). A male and female Runx2^{+/-} mouse were mated for 24hrs. 14.5days in pregnancy, the female mouse was sacrificed. Fetus were collected and fetal livers isolated. Adult female WT mice were sub lethally irradiated (1000 cGy) and reconstituted via tail vein injections of fetal livers. Once the immune systems were reconstituted, Runx2 deficiency was verified using Real Time PCR (RTPCR) and flow cytometry. Primers for genotyping: Neo3F: AAG ATG GAT TGC ACG CAG GTT CTC, CbfaDB: CAC GGA GCA CAG GAA GTT GGG. Runx2 antibody from cell signaling (D1L7F), Anti-rabbit IgG (H+L), F(ab')₂Fragment (Alexa Fluor® 488 Conjugate) #4412 was used as a secondary antibody.

In vivo Bioluminescence Imaging

Before imaging, mice were anesthetized with isoflurane and i.p. injected with 100 mL of 20 mg/mL D-Luciferin (Xenogen Corp.). After 8 min, animals were imaged using an IVIS 200 system

(Xenogen), according to the manufacturer's instructions. Living Image software (Xenogen) was used to analyze data. Regions of interest were manually selected and quantification was reported as the average photon flux within regions of interest. The bioluminescence signal detected was represented as photons/s/cm² /sr.

Adoptive T cell transfer

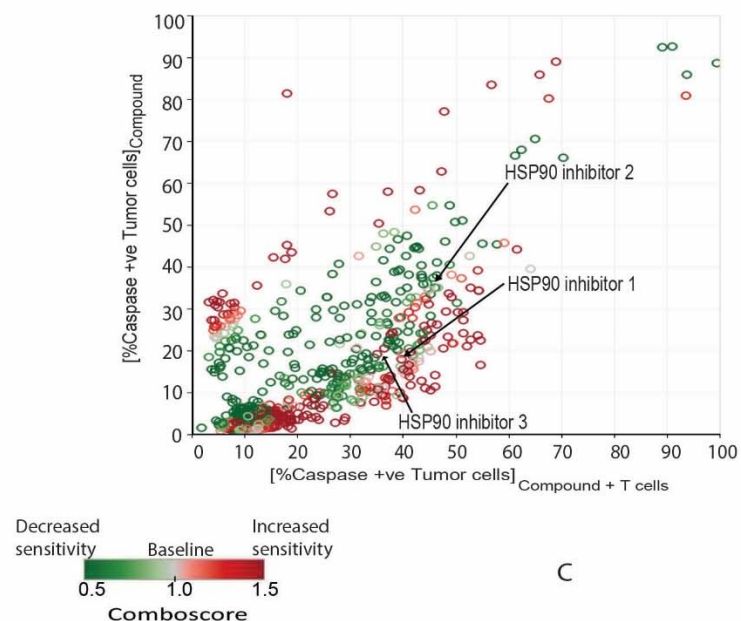
Nine days before ACT, splenocytes from pmel-1 TCR/Thy1.1, Runx2^{+/-} pmel-1 TCR/Thy1.1 and Runx2^{-/-} pmel-1 TCR/Thy1.1 transgenic mice were harvested and infected with a retroviral vector encoding a modified firefly luciferase gene and green fluorescent protein as previously described. After sorting based on green fluorescent protein expression, luciferase-expressing pmel-1 T cells were used for ACT. Wild-type (WT) were subcutaneously implanted with either 500000 B16 cells (day 0). On day 6, lymphopenia was induced by administering a nonmyeloablative dose (350 cGy) of radiation. On day 7, 1e10⁶ luciferase-expressing pmel-1 T cells were adoptively transferred into tumor-bearing mice (n ¼ 3–5 per group), followed by intravenous injection of hgp100 peptide-pulsed bone marrow–derived dendritic cells (DC) generated as previously described. Recombinant human IL-2 was intraperitoneally administered for 3 d after T-cell transfer (1.2e10⁶ IU once immediately after T-cell transfer and 6e10⁵ IU twice daily for the next 2 days). Tumor sizes were monitored every 2 days.

Statistical analysis.

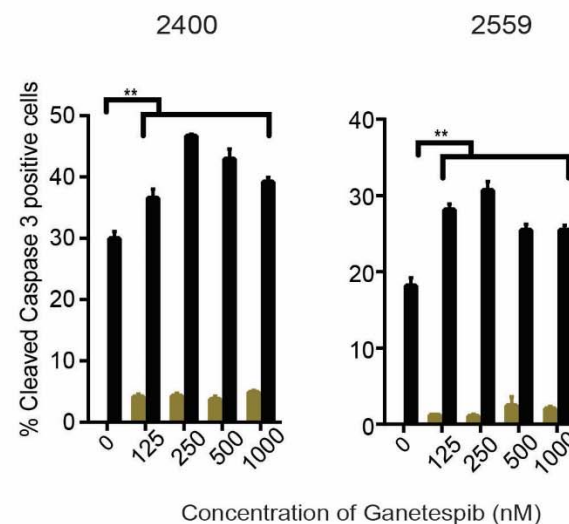
The data were represented as mean ± SEM. Comparisons of differences in continuous variables between 2 groups were done using unpaired Student t tests. Comparisons of differences in continuous variables within a group (DMSO vs peptide stimulation) were done using paired Student t tests. Differences in tumour size and T-cell numbers among different treatments were evaluated by ANOVA repeated-measures function. P values are based on 2-tailed tests, with P < 0.05 considered statistically significant. Graphs were generated using GraphPad Prism 6 and Tableau. Statistical analyses were performed using GraphPad Prism 6.

Appendix

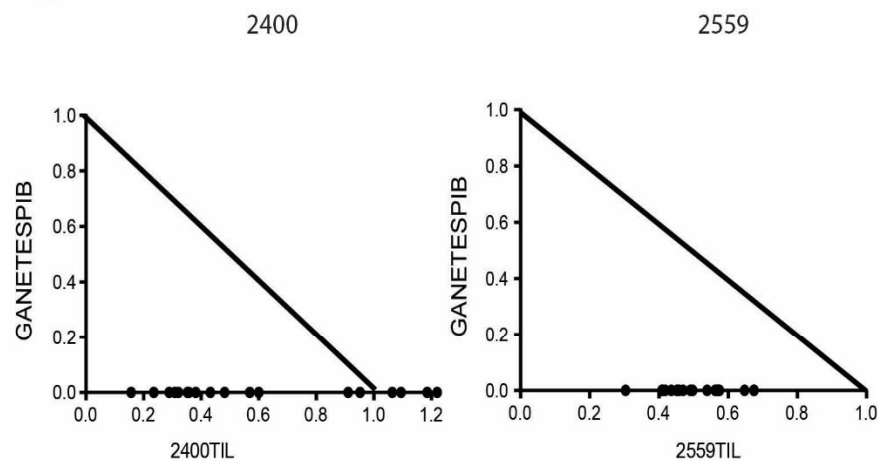
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B

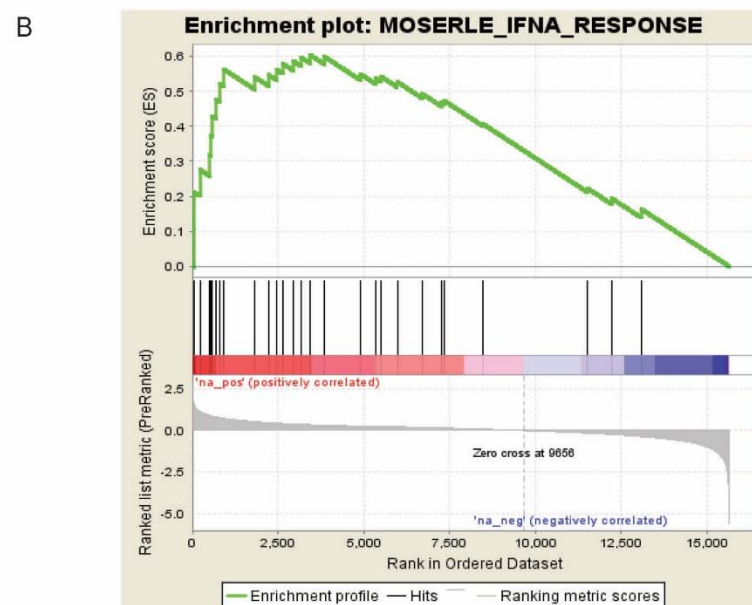
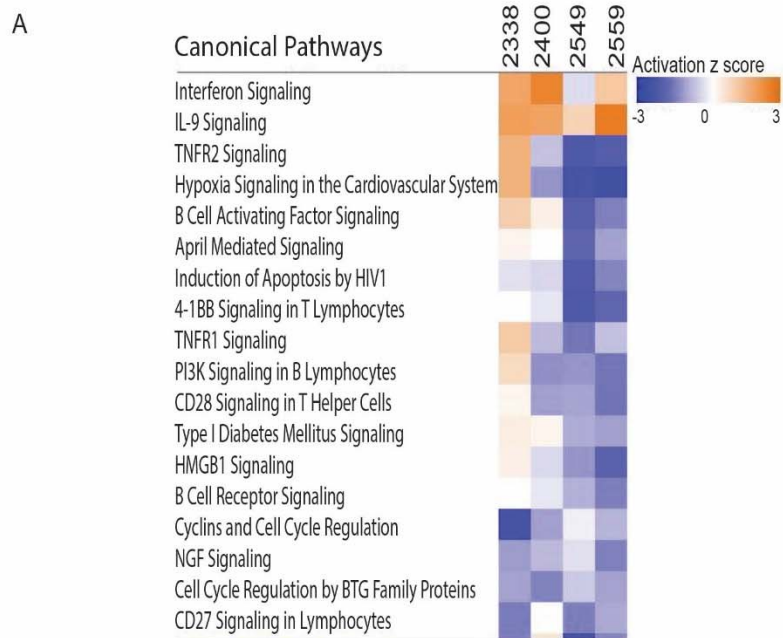


C



Supplemental Figure 1: HSP90 inhibition enhances T cell mediated killing of melanoma cells. (A) Tableau depiction of screen results from patient derived cell line 2338. HSP90 inhibitors 1 - 17-DMAG, 2 – BIIB021 and 3 -17-AAG are highlighted. (B) Cleaved caspase 3 percentage following treatment with varying concentrations of ganetespib and autologous TILs in human melanoma cell lines 2400 and 2559. (C) Normalized isobolograms depicting the synergism between ganetespib and T cell killing. Combination indexes < 1 indicate synergy.

**P < 0.01. Data are a representation of at least two independent studies.

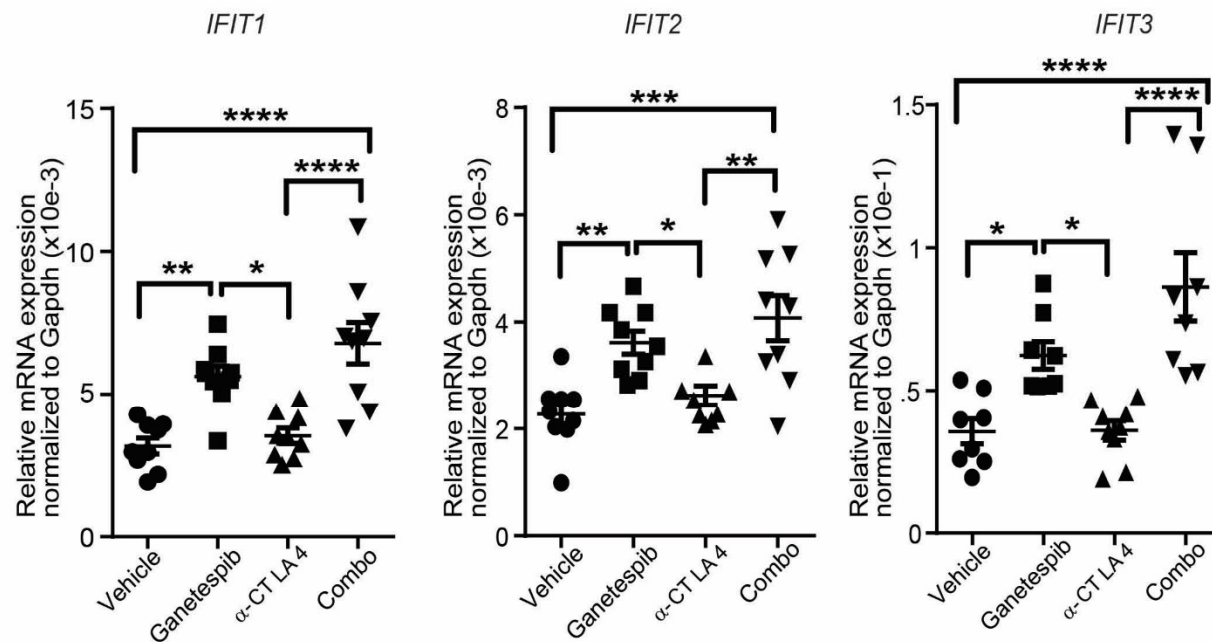


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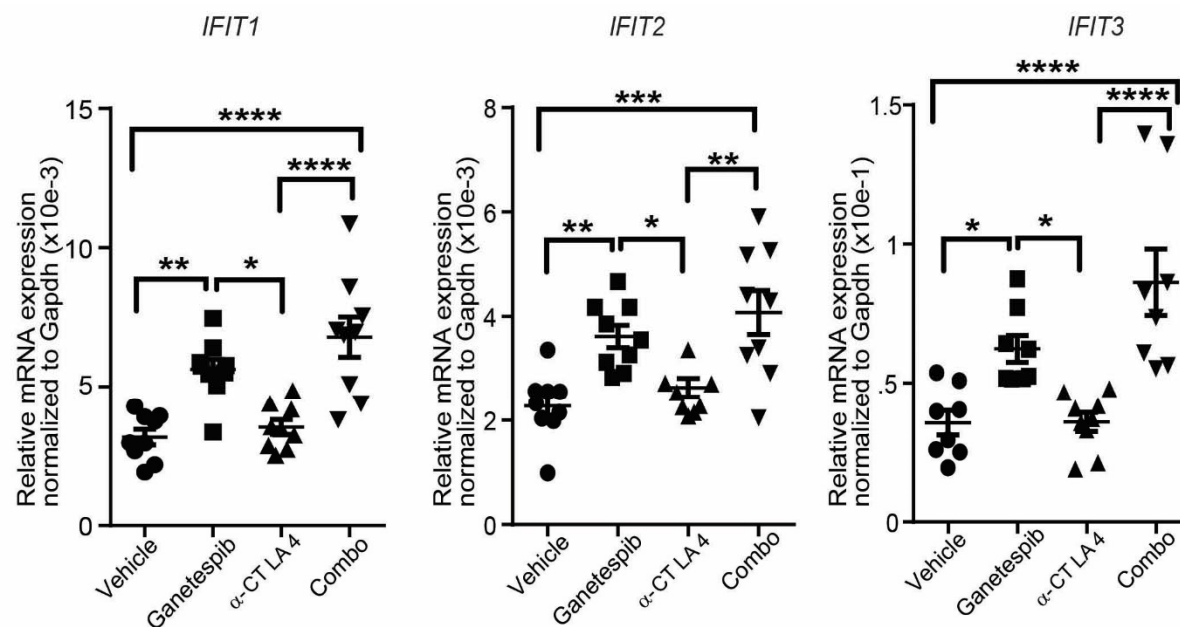
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row_0	IFIT3	44	1.60714376	0.10464722	Yes
row_1	IFIT2	45	1.604997277	0.21196944	Yes
row_2	EPSTI1	223	1.127469301	0.27602237	Yes
row_3	GBP1	512	0.895565569	0.31745812	Yes
row_4	RTP4	548	0.880248189	0.37407613	Yes
row_5	IFIT1	586	0.860438883	0.42924145	Yes
row_6	DDX60	705	0.807804108	0.47569853	Yes
row_7	MX1	788	0.779251099	0.52255243	Yes
row_8	IFIH1	921	0.734060228	0.56318164	Yes
row_9	ZC3HAV1	1834	0.53478986	0.5405214	Yes
row_10	HERC5	2221	0.475829691	0.5476128	Yes
row_11	DDX58	2476	0.445198894	0.56111157	Yes
row_12	SAMD9	2639	0.427447706	0.5793167	Yes
row_13	TRIM22	2938	0.400018007	0.58697575	Yes
row_14	DDX60L	3164	0.379113734	0.59791327	Yes
row_15	IFI44	3441	0.356272995	0.60405654	Yes
row_16	OAS2	3849	0.327158958	0.59986144	No
row_17	TNFSF10	4901	0.260549664	0.5499595	No
row_18	USP18	5340	0.236566901	0.537721	No
row_19	CXCL10	5504	0.229704812	0.54263943	No
row_20	RSAD2	5973	0.219200432	0.52731794	No
row_21	SAMD9L	6695	0.188416034	0.4937315	No
row_22	IFIT5	7257	0.160109058	0.4685014	No
row_23	IFI16	7352	0.155639321	0.47288722	No
row_24	IFI44L	8455	0.087456793	0.408144	No
row_25	OAS1	11513	-0.164081007	0.22329223	No
row_26	STAT1	12228	-0.242892444	0.19379687	No
row_27	IFITM1	13095	-0.367514521	0.16289796	No

Supplemental Figure 2: Interferon response genes are upregulated following HSP90 inhibition.

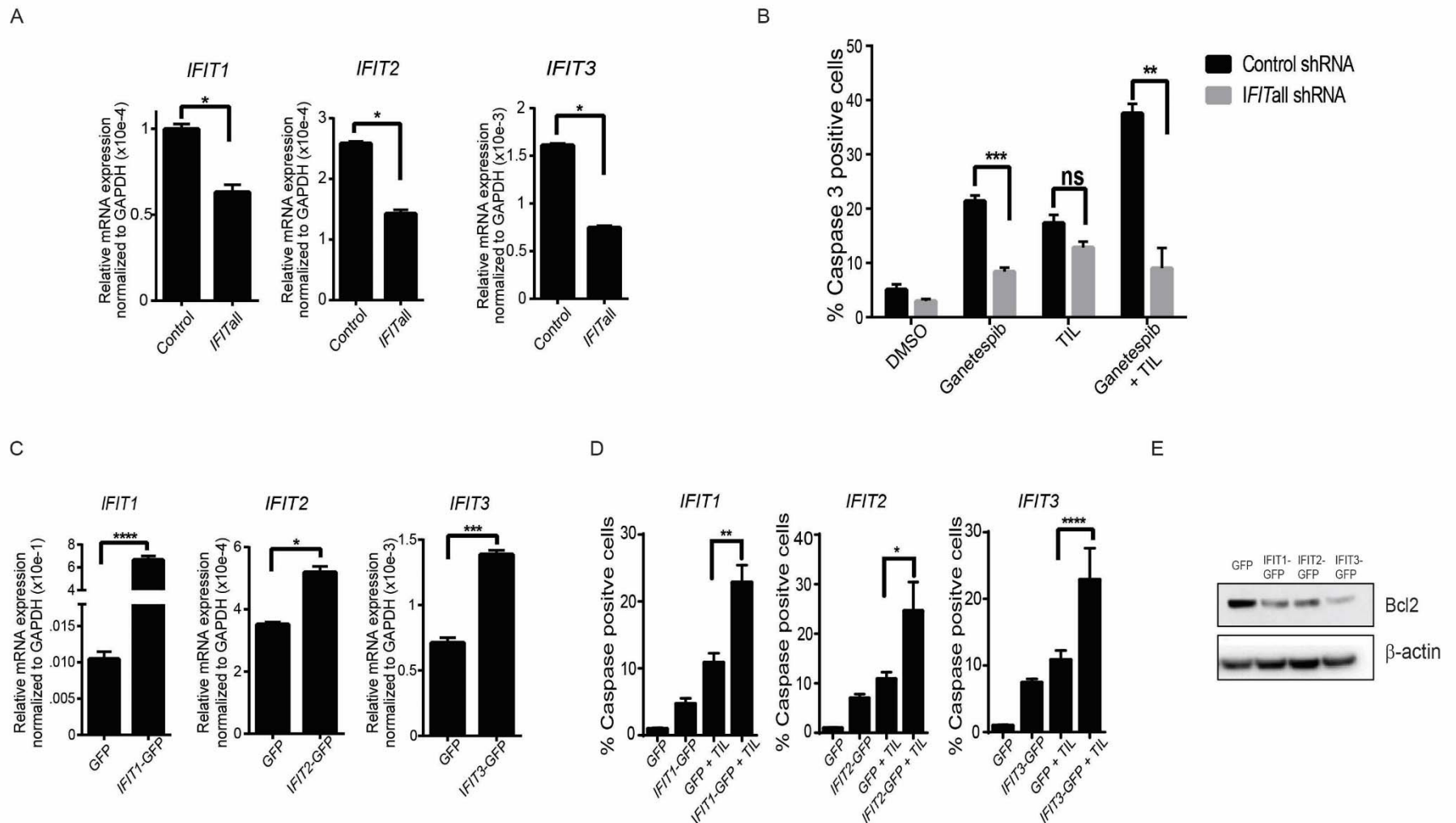
(A) Hierarchical clustering of canonical pathways across samples using comparison analysis in the Ingenuity pathway analysis software. Heat map shows interferon signaling the most consistently upregulated pathway across all four cell lines. (B)&(C) GSEA shows interferon response genes upregulated following treatment with ganetespib.



Supplemental Figure 3: Interferon response genes are upregulated following HSP90 inhibition. Graphs depict the in vivo expression of IFIT genes by qRT-PCR. Combo = ganetespib + α-CTLA4. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. n = 9 mice.



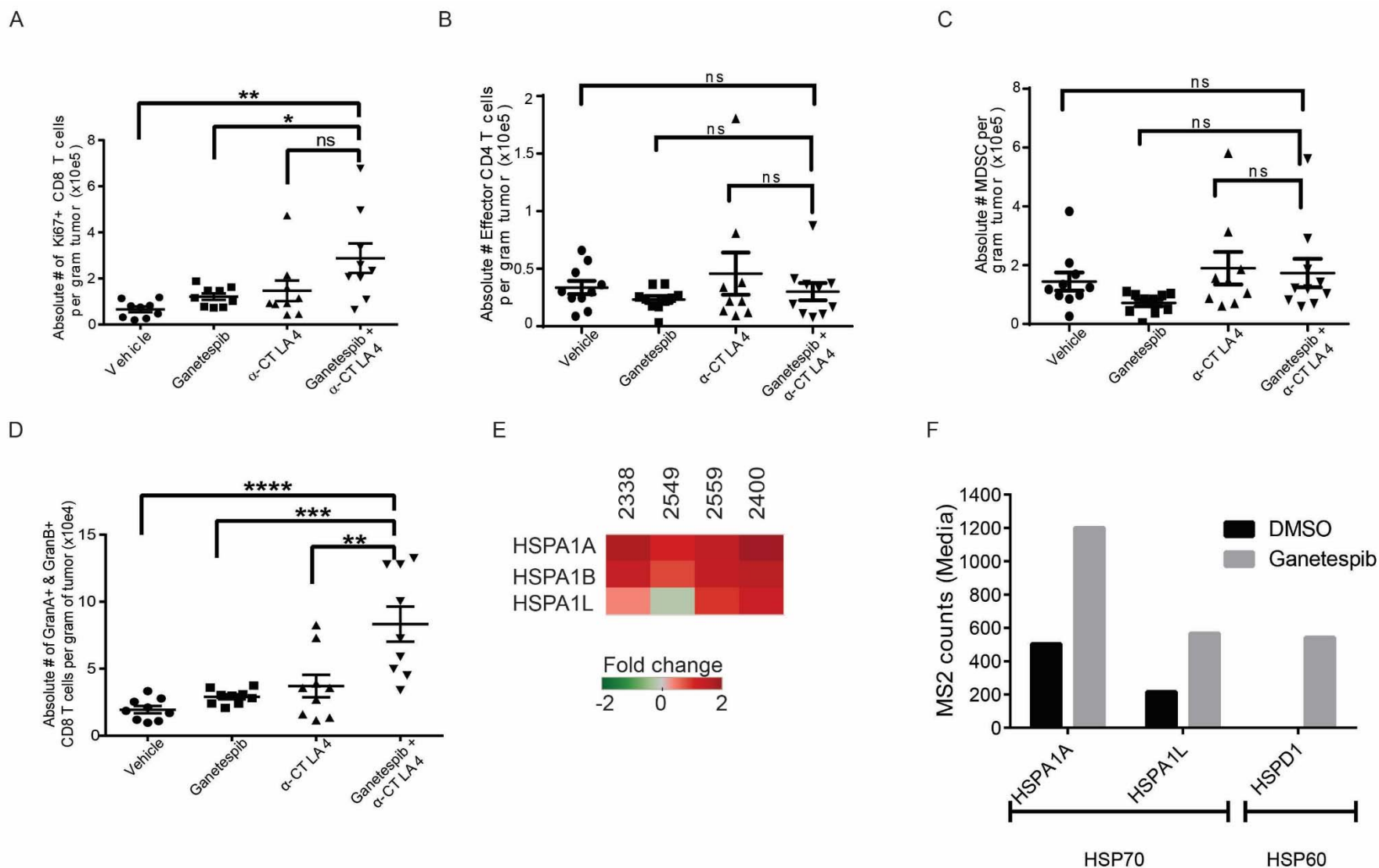
Supplemental Figure 3: Interferon response genes are upregulated following HSP90 inhibition. Graphs depict the in vivo expression of IFIT genes by qRT-PCR. Combo = ganetespiib + α-CTLA4. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. n = 9 mice.



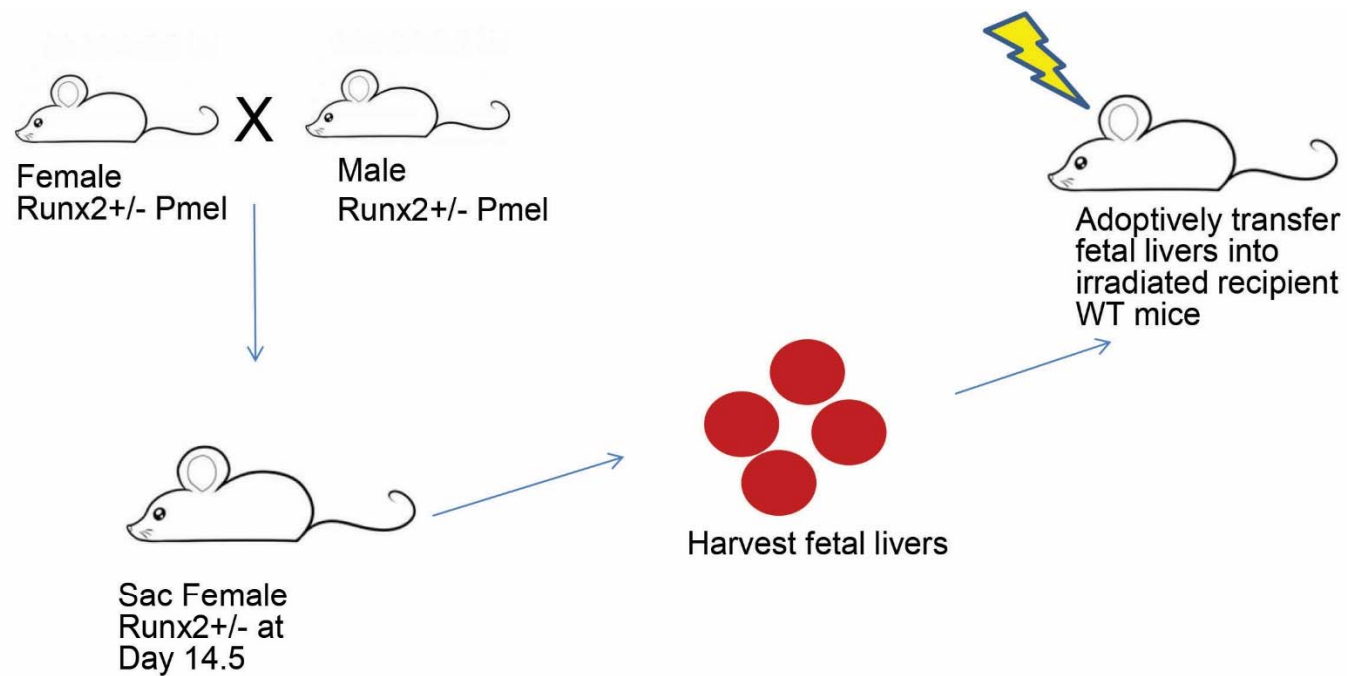
Supplemental Figure 5: The IFIT genes are essential for the enhanced T cell killing of tumor cells following HSP90 inhibition.

(A) qRT-PCR to verify silencing of IFIT1, IFIT2 and IFIT3 in 2549. Control = 2549 transduced with scrambled shRNA and IFITall = 2549 transduced with IFIT1, IFIT2 and IFIT3 shRNAs simultaneously. (B) 2549 Control and IFITall cell lines treated with ganetespib at 250nM, co-cultured with autologous T cells and assayed for cleaved caspase 3. (C) qRT-PCR verifying overexpression of IFIT1, IFIT2 and IFIT3 over GFP control in 2549. (D) 2549 GFP and IFIT overexpressing cell lines co-cultured with autologous T cells and assayed for cleaved caspase 3. (E) Western blots showing a decrease in BCL2 protein after overexpression of IFIT1, IFIT2 and IFIT3.

*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

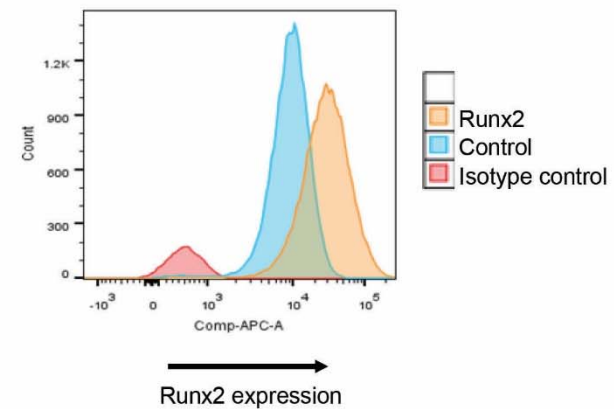
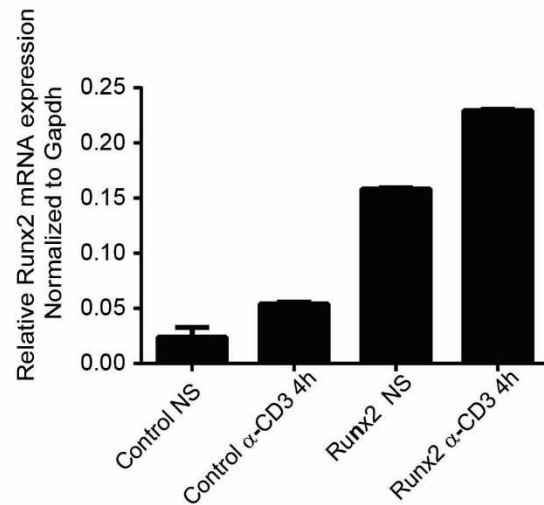


Supplemental Figure 6: The combination of HSP90 inhibition and anti-CTLA4 treatment modulates the immune cell population in the tumor (A) Absolute number of Ki67 positive CD8 T cells infiltrating the tumor normalized to tumor weight. (B) Absolute number of effector CD4 T cells (FOXP3 negative) infiltrating tumor normalized to tumor weight (C) Absolute number of MDSCs infiltrating tumor normalized to tumor weight. (D) Absolute number of granzymes A and B double positive CD8 T cells infiltrating the tumor normalized to tumor weight. (E) HSP70 expression upregulated at mRNA level following ganetespi treatment. (F) HSP70 and HSP60 secreted following treatment with ganetespi in melanoma cell line 2338. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. $n = 9$ mice.

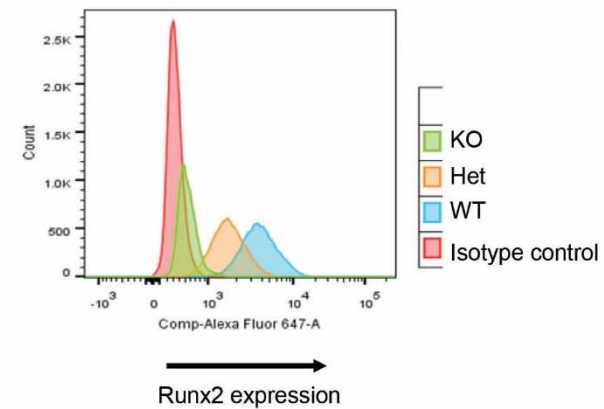
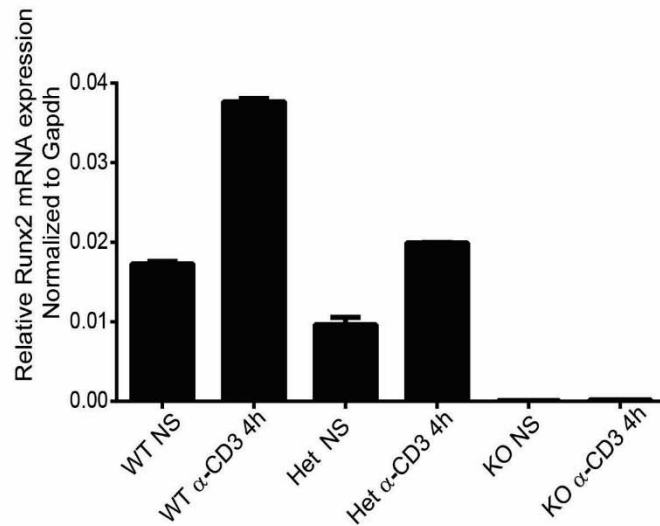


Supplemental Figure 7: Fetal liver transplant procedure for the generation of Runx2 heterozygous and homozygous knockout Pmel cells

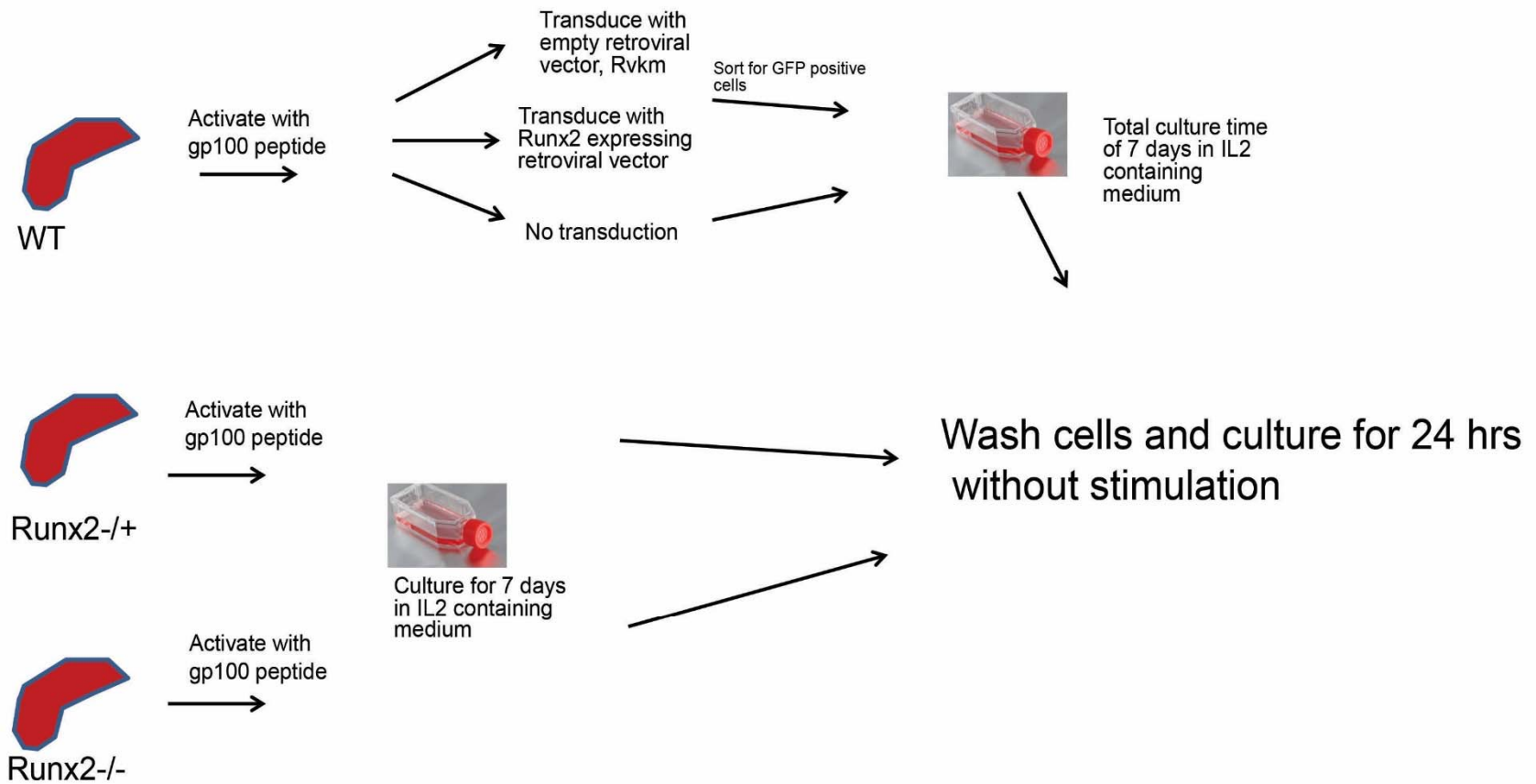
A



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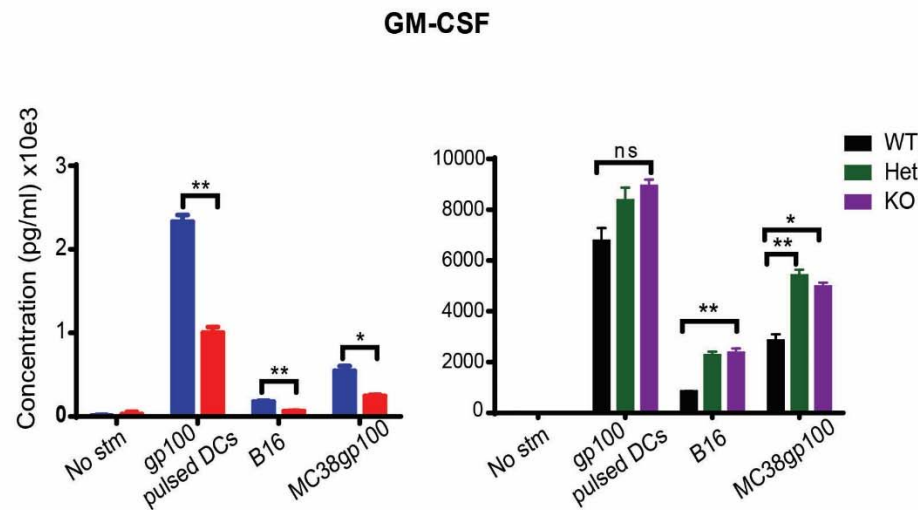


Supplemental Figure 8: Verification of Runx2 expression in Runx2 perturbed Pmel T cells. A) Verification of Runx2 overexpression by quantitative real time PCR and flow cytometry the presence of no stimulation (No stm) and plate bound anti-CD3. B) Verification of Runx2 deficiency by quantitative real time PCR and flow cytometry the presence of no stimulation (No stm) and plate bound anti-CD3. Control = GFP control, Runx2 = Runx2 overexpression, WT = Untransduced control, Het = Runx2 heterozygous, KO = Runx2 homozygous.

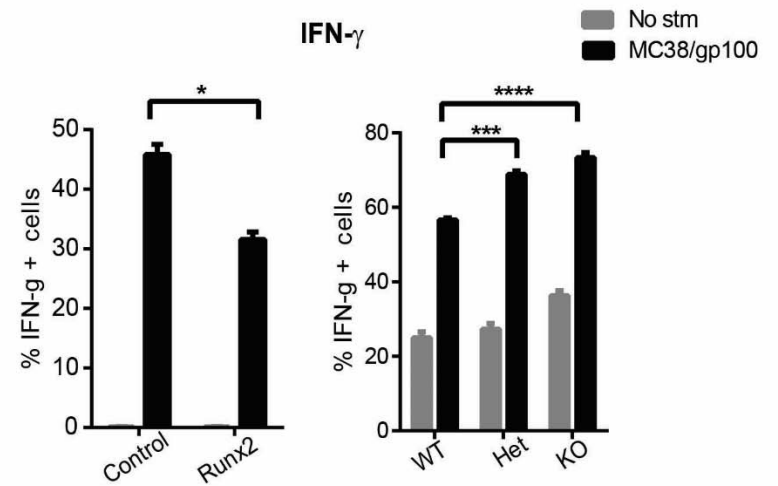


Supplemental Figure 9: Transduction and culture conditions for Pmel T cell

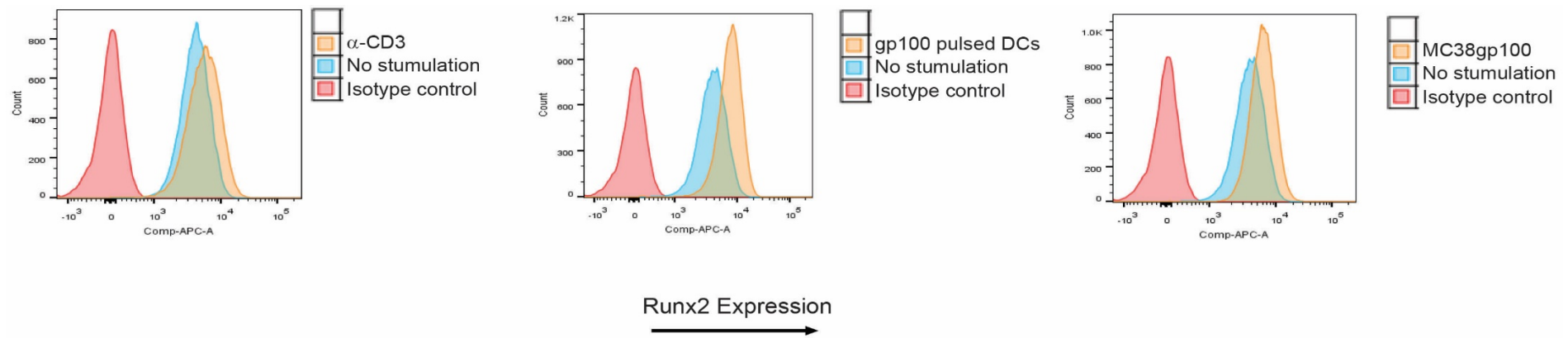
A



B



Supplemental Figure 8: Runx2 expression promotes effector functions reminiscent of terminal effector CD8 T cells . A) GM-CSF secretion by Pmel T cells in the presence of no stimulation (No stm), gp100 pulsed DCs B16 and MC38gp100 tumour cells. B) Percentage of IFN- γ positive cells measured by flow cytometry. Control = GFP control, Runx2 = Runx2 overexpression, WT = Untransduced control, Het = Runx2 heterozygous, KO = Runx2 homozygous. *P < 0.05, **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are a representation of at least two independent studies



Supplemental Figure 11: Runx2 expression expression by flow cytometry under different stimulatory conditions

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